

تحقیق در مورد پادزهر کزاز

STUDIES ON ANTI-TETANUS ANTIBODIES

by

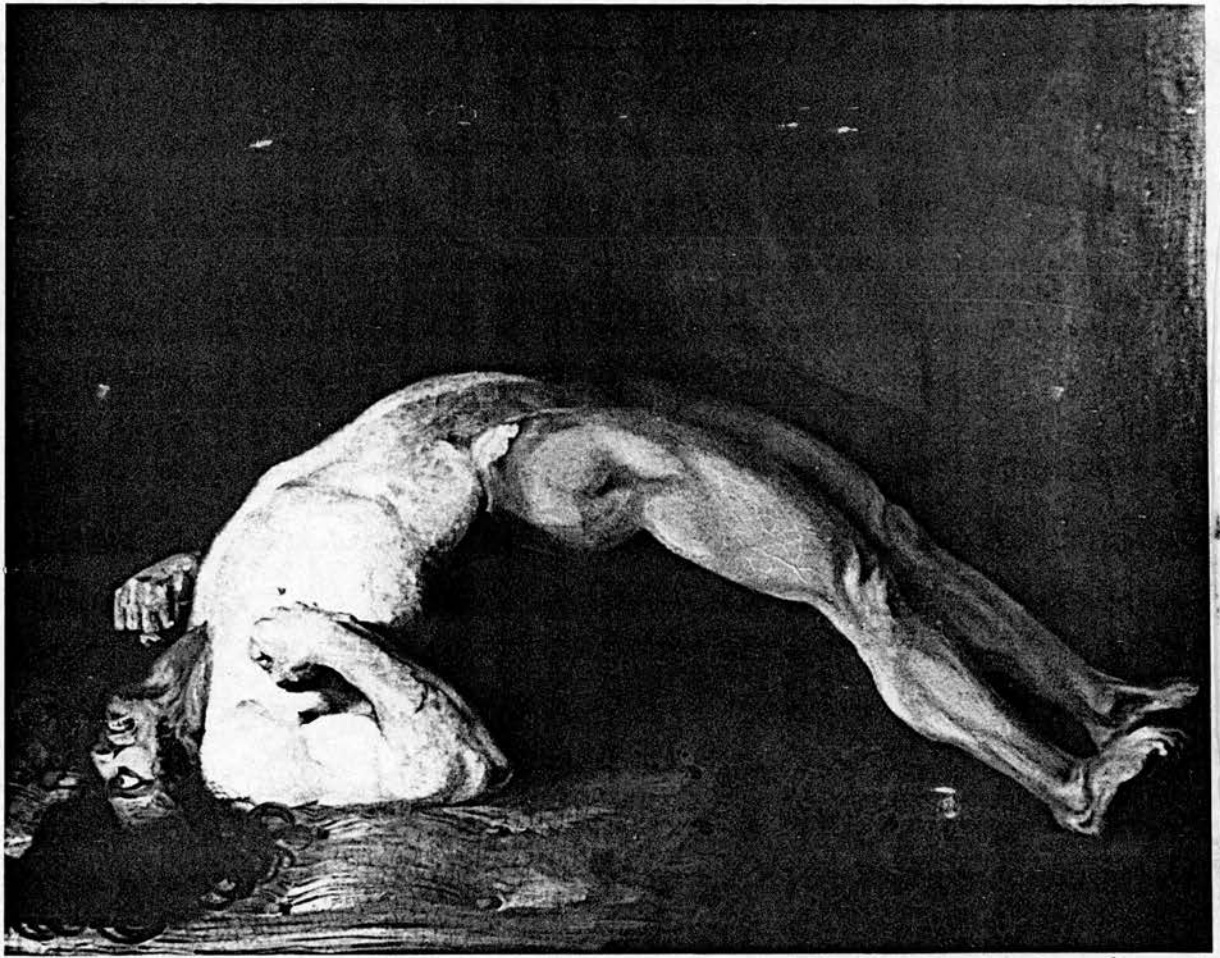
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Frontispiece. Tetanus. Sketch in oil by Charles Bell, of opisthotonos taken from three soldiers, who were wounded at Corunna and brought to Portsmouth. They died successively from gunshot fracture of the skull. (Photograph provided and reproduced by courtesy of the Royal College of Surgeons, Edinburgh).

به دخترهایم

TO MY DAUGHTER

YASMIN

We shall not cease from exploration  
And the end of all our exploring  
Will be to arrive where we started  
And know the place for the first time.

T.S. Eliot (1942)



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# DECLARATION

I declare that I have composed and written this thesis and that the work described in this thesis was entirely my own and performed by me unless otherwise acknowledged.

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## ABSTRACT

Studies were undertaken to produce and characterize enriched anti-tetanus antibodies with potential therapeutic use.

A sensitive enzyme immunoassay (ELIZA) was developed for the quantitation of antitetanus antibodies. This technique was used to measure antibody levels in the sera of immunized donors, in crude and purified tetanus-IgG preparations and for screening human and mouse monoclonal antibodies to tetanus toxoid. The assay was capable of measuring antibody levels as low as  $5 \times 10^{-4}$  IU/ml. The antigen coated-plates prepared for this study had a shelf life of at least one year. By inclusion of a third step involving antibodies to mouse Ig-isotypes, a sandwich enzyme immunoassay (SEI) was developed, which enabled the determination of the Ig-isotype of antitetanus antibodies. The isotype specificity of this assay was confirmed by studies on Protein-A Sepharose fractions of mouse antiserum.

The homogeneity of commercial tetanus toxoid preparations was studied using gel filtration. The Wellcome tetanus vaccine proved to be extremely heterogeneous; the Connaught tetanus toxoid was purer and had higher toxoid activity.

An immunoaffinity technique was developed to isolate antibodies with specificity for tetanus toxin from human plasma and from polyvalent tetanus-IgG (Cohn Fraction II). Studies were undertaken to establish the optimum conditions for the preparation of the immunoaffinity column; the factors which were found to influence the performance of the columns are discussed. The optimized column was stable in storage and could be used repeatedly to isolate tetanus antibodies without loss of capacity. The specific antibody was extracted from the crude material by passing it through the column

containing the immobilized antigen. Desorption from the column was carried out at acid pH. Immunological and biochemical properties of the preparations are presented. The affinity purified preparations had specific neutralizing activities up to 182 IU per mg of IgG representing up to 108-fold purification.

Studies were carried out on the immune response of CBA and BALB/c mice to two tetanus toxoid preparations to determine whether the antitetanus response was isotype restricted, whether there were differences in the immunogenicity of the two toxoids, and finally to establish the ontogeny of the response. An interesting finding was the presence of naturally occurring antibodies which bound to the tetanus toxoids in both strains of mice; these were of the IgA and IgM isotypes. Furthermore, the purer toxoid preparation elicited a more rapid and pronounced response than the crude vaccine. After a primary challenge, the antibody responses to tetanus toxoid were detected in all IgG subclasses except that of IgG<sub>2b</sub>, and on boosting the antibody response was mainly of IgG<sub>1</sub> subclass. Both strains of mice exhibited similar responses.

A number of murine monoclonal antibodies were produced. These were found to be of IgG<sub>1</sub> isotype using the SEI assay. A stable human cell line (designated ES-12) secreting monoclonal antibodies to tetanus toxoid was obtained. The antibody produced by this cell line protected mice against the effect of tetanus toxoid. In both in vivo and in vitro assays, this monoclonal had an antitetanus antibody level of 2.5-3.0 IU/ml.

The potential use and limitation of both immunoaffinity purified and monoclonal antitetanus antibodies in human prophylaxis and therapy are discussed.

## ABBREVIATIONS

Ab	Antibody
Ag	Antigen
Ap	Alkaline phosphatase
ATS	Anti-tetanus serum
BSA	Bovine Serum Albumin
Conn-TT	Connaught tetanus toxoid
ELISA	Enzyme Linked Immunosorbant Assay
FCS	Foetal calf serum
g	Gram
HA	Haemagglutination Assay
HAT	Hypoxanthine-aminopterin-thymidine
HIA	Haemagglutination Inhibition Assay
hr	Hour
HSIG	Human serum globulin
HT	Hypoxanthine-thymidine
Ig	Immunoglobulin
im	Intramuscularly
ip	Intraperitoneally
iv	Intravenously
LMW	Low Molecular Weight
LPS	Lipopolysaccharide
mA	Milliamperes
McAb	Monoclonal antibody
NHS	Normal Human Serum
NMS	Normal Mouse Serum
O/N	Overnight
PBL	Peripheral blood lymphocytes
PBS	Phosphate Buffered Saline
PEG	Polyethylene glycol
RIA	Radioimmunoassay
RT	Room Temperature
SAS	Saturated ammonium sulphate
Sec	Section
SEI	Sandwich Enzyme Immunoassay
TCM	Thymocyte culture medium
TIG	Tetanus immunoglobulin
TT	Tetanus Toxoid
WELL-TT	Wellcome tetanus toxoid

## A. GENERAL INTRODUCTION

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### A.1 History of Immunoglobulin Use

History of immunoglobulin (Ig) use began in 1890 when von Behring and Kitasato announced that the serum of rabbit and mice that had been immunized against tetanus had the property of neutralizing the effect of the toxic substance produced by the tetanus bacillus. This property of the serum was so durable that it remained active when the serum from an immunized animal was transferred to a non-immunized animal. This progressed through the clinical use of animal antitoxins in the prophylaxis and treatment of human disease. The diphtheria antitoxin prepared by Ehrlich (1897) constituted the first biological standard.

The development of Ig-therapy has been one of the mainstreams of the science of immunology over the last 40 years. In the early 1920's, the characterization of the pneumococcal polysaccharides prompted extensive studies (reviewed by Janeway, 1970), which led to the purification of antipneumococcal antibodies from animal sera for therapeutic use. During the 1930's purified heterologous anti-pneumococcal antibodies were widely used in the treatment of pneumococcal infection. However these preparations which were purified from horse and later rabbit antiserum by salt fractionation had major drawbacks, for they elicited severe allergic reactions when repeatedly administered. Some of the animal antisera available and used for passive immunity are listed in Table 3. It should be noted that the only antisera available in many countries to tetanus and rabies are of equine origin. The application of most of the animal preparations were abandoned once antibiotics became available.

Although progress in antibiotic research has been impressive at the biochemical and microbiological levels, the mortality from pneumococcal pneumonia is still close to 10% despite the exquisite sensitivity of this organism to Penicillin G. Adult or neonatal bacterial meningitis generally due to encapsulated micro-organisms carries a mortality rate close to 40%, and Gram negative sepsis has a mortality of 30% - 50% even under optimal antibiotic management (Waldvogel, 1981). These and other therapeutic limitations indicate that other treatment modalities will continue to be necessary to prevent or treat the explosive growth of micro-organisms during infection. This could be achieved by devising methods which can increase the clearance of pathogenic organisms or their toxic products. Active and passive immunization have been the strategies most extensively used in this respect.

Human immune serum globulin is a valuable and frequently prescribed biological used to provide passive immunity against microbial agents, toxins, or cells. Immunoglobulin is mostly used for the short term preparation of certain bacterial or viral diseases when active immunization is unavailable (i.e. hepatitis A) or when active immunization has not been given before exposure (i.e. tetanus and rabies). It is also used on a continuous basis in replacement therapy for persons with antibody immunodeficiency.

Two types of immunoglobulin preparations are available: standard human immune serum globulin (HISG) for general use (Table 1) and specific immunoglobulins with a known antibody content for specific illnesses (Table 2).

## A.2 Some Properties of Immunoglobulins

The immunoglobulins (reviewed by Fahey, 1970; Janeway, 1970; Spiegelberg, 1974; Stiehm, 1979) are the proteins of the plasma and tissue which have antibody activity. Although there are five classes of immunoglobulins - IgG, IgM, IgA (secretory IgA), IgD and IgE, only IgG is present in significant quantities in HISG.

IgG is a glycoprotein with a molecular weight of 150,000 daltons distributed equally between the serum and the tissue. The IgG molecule is Y-shaped with two combining sites, one at the end of each arm (Fab). It is made up of two heavy (gamma) chains and two light (kappa or lambda) chains held together by disulphide bonds and weak covalent forces. Its structure is illustrated in Fig.1.

There are 4 subclasses of human IgG, IgG<sub>1</sub>, IgG<sub>2</sub>, IgG<sub>3</sub> and IgG<sub>4</sub> comprising about 70%, 15%, 10% and 5% respectively of the total IgG. All normal persons have IgG molecules of all subclasses. These subclasses differ antigenically and exhibit differences in biological activity.

IgG has a mean half-life of 25 days, the longest half life of any plasma protein. The synthetic rate of IgG is 35 mg per kilogram of body weight per day. IgG metabolism is regulated by the serum level of IgG, so there is an increase in catabolic rate with high levels of IgG and a decrease at low levels. Thus, the IgG half-life of patients with agammaglobulinemia is prolonged to 35 - 40 days.

IgG is made primarily in plasma cells that have differentiated from circulating B-lymphocytes. These are located in central lymphoid tissues (lymph nodes, bone marrow, spleen and liver), and peripheral lymphoid tissues (secretory glands, intestinal walls). IgG destruction



occurs within granulocytes (after phagocytosis of IgG-coated bacteria and particles) in the reticuloendothelial system. IgG readily crosses the placenta during late pregnancy, providing passive immunity to the newborn for about six months.

IgG which comprises about 20% of total plasma protein and 80% of the serum immunoglobulin, is the chief component of the body's serological defences. It contains most of the antibacterial, antiviral, antiprotozoal and antitoxic activity of the serum. Many cells (i.e. lymphocytes, macrophages, killer cells) have a receptor for the nonantibody (Fc) portion of IgG, permitting attachment of IgG to these cells. The presence of antibody on target cells (tumour cells, heterologous erythrocytes, allogeneic lymphocytes) may permit antibody-dependent cytotoxicity by some lymphocytes, neutrophils and macrophages.

IgG can also activate the complement systems, promote opsonization (i.e. the recognition of bacteria by the phagocyte receptors), and participate in antibody-dependent cytolytic reactions. It should be pointed out that certain IgG subclasses (i.e. IgG<sub>1</sub> and IgG<sub>3</sub>) are more cytotoxic and more able to fix complement than others (Shakib et al. 1980).

### A.3 Clinical Applications

Indications for the therapeutic use of immunoglobulins have been extensively reviewed in the literature (Janeway, 1970; Stiehm, 1979; Nydegger, 1981, McClelland et al. 1984 and McClelland, 1984) and will be briefly summarized here. Tables 1, 2 and 3 provide examples of diseases in which immunoglobulins (HSIG, specific or heterologous) have been used.

The use of human Ig preparations for passive immunization began with the studies of McKhann and co-workers (1935), who used ammonium sulphate to isolate Ig fractions from placental extracts. The major advance in this field came with Cohn and co-workers' method of cold ethanol fractionation of Ig from human plasma (Cohn et al. 1946). These preparations have been widely used for the treatment or prevention of infectious diseases, and were also given to patients with congenital or acquired antibody deficiencies. However, it was soon recognized that intravenous injection of these preparations frequently gave rise to adverse reactions. Efforts to eliminate these adverse reactions by further purification and screening tests in animals ended in failure, and therefore it was accepted that these preparations should be restricted to intramuscular administration (Janeway, 1970).

#### A.3.1 Indications of Human Serum Globulin (HSIG)

Many antibody immunodeficiencies are treated successfully with repeated intramuscular injections of HISG (see table for references). The widest use of HISG is in the prevention of hepatitis A. Its efficacy in this respect has been repeatedly demonstrated since Stokes and Neeffe (1945) showed that it prevented an epidemic in a children's summer camp. Indications for the use of HISG for prevention of hepatitis B are limited to the rare situations where hyperimmune anti-hepatitis B antibody preparations (HBIG) are not available (Seeff et al. 1979, review); its use in the prevention of non-A non-B hepatitis however seems promising.

Administration of immunoglobulin in measles is becoming limited by the growing proportion of vaccinated children. Nevertheless, the preparation can be useful for non-vaccinated children exposed to

measles, and followed by vaccination. Rubella prevention by HISG is controversial because of its unreliable efficacy, but high doses of rubella specific antibody (RIG) was found to prevent the infection (Schiff, 1969). Finally, HISG has shown to offer incomplete and short-term protection against the paralytic poliomyelitis syndrome (reviewed by Stiehm, 1979). HISG was used extensively in the prevention of poliomyelitis before the development of specific vaccines.

#### A.3.2 Specific Antibody Preparations and their Use

These limited indications have prompted many researchers to develop potent and specific antibody preparations against particular viruses and bacteria. During the last decade HISG preparations have been partly replaced in clinical use by specific Ig. Preparation of the specific Ig is identical to that of HISG (Cohn fractionation technique), except selected donors with high natural titres against specific antigen or hyperimmunized donors are used, whereas HISG is isolated from the plasma of non-selected donors.

It is beyond the scope of this introduction to discuss in detail the various indications for hyperimmune serum globulins (for review, see again Stiehm, 1979; McClelland et al. 1984). A list of diseases for which specific immunoglobulins are available can be seen in Table 2, and only a brief overview of the subject will be given.

Relative indications of HBIG for hepatitis B are presently limited to subjects accidentally contaminated by HB<sub>S</sub>-Ag containing blood or secretions, neonates from mothers positive for HB<sub>S</sub>-Ag, and spouses of HB<sub>S</sub>-Ag carriers (Reesnik et al. 1979 and Tabor, 1980).

Administration of zoster immune globulins, or when available of plasma

from a patient recovering from herpes zoster, will prevent the complications of varicella in exposed, susceptible, immuno-suppressed patients (Zia et al. 1980 and 1983). Rabies immune globulins have become accepted standard therapy in conjunction with vaccination in the prevention of clinical disease after bites due to rabid animals (Mertz, 1982). Vaccinia immune globulin has been shown to be effective in protecting household contacts in case of smallpox (Stiehm, 1979). The apparent eradication of smallpox has prevented the various indications for the time being. Use of immune globulin from vaccinated adults in the treatment of pertussis (Whooping cough) has been reported, if given early in the incubation period. However, given during the coughing stage it has been ineffective. Mumps immune globulins which is indicated for postpubertal males who do not want to risk sterility has not been shown to be effective (Stiehm, 1979). Rh (D) immunoglobulin (RhIG) is used to prevent sensitization of Rh (D)- negative individuals who have received Rh (D)-positive blood as a result of delivering an Rh(D)-positive infant or fetus, or as a result of a transfusion accident (Diamond, 1970 and Smith et al. 1972). Finally, the toxin-neutralizing properties of animal antisera have been used for several years in the prevention or early treatment of tetanus, diphtheria and botulism (Table 3). Although highly effective at the preventive stage, their clinical efficacy during disease has sometimes been disputed. Tetanus and TIG will be discussed in detail later in this chapter.

### A.3.3 Clinical Use of Monoclonal Antibodies

The introduction of the hybridoma technology by Kohler and Milstein (1975) has revolutionized the diagnosis and possible treatment of many human diseases. There is already available a wide range of monoclonal antibodies of both proven and potential diagnostic importance while many therapeutically important reagents are under development (Table 4). For review, see McClelland (1984), James et al. (1984) and McMichael (1981). The production of monoclonal antibodies, their advantages and the problems associated with them, are discussed in Chapter 6.

## A.4 Tetanus

### A.4.1 History of Tetanus

Tetanus has long been recognized as a disease of man and animals. Nicolaier (1885) suggested that the organism causing tetanus was not distributed throughout the body but confined to the wound of entry. In 1854 Simpson had already observed that the symptoms of tetanus were similar to those of strychnine poisoning. The work of Nicolaier was extended by Kitasato in 1889 who isolated the tetanus bacillus. In the next year von Behring and Kitasato (1890) demonstrated the presence of the neurotoxin in culture filtrates of the organism. They also found that the administration of many small doses of the toxin in animals induced immunity against it. Demonstration of antitoxin in the serum of animals so immunized, together with similar findings with the toxin of Corynebacterium diphtheriae, laid the foundations for the humoral theories of immunity as well as for the vaccine prophylaxis and serum therapy of many diseases (Smith, 1975).

#### A.4.2 Pathogenesis

Tetanus is usually the result of contamination of a wound with Clostridium tetani (an anaerobic bacillus), the spores of which are widely distributed in soil and in the intestinal tracts of man and animals (reviewed by Willis, 1969; Smith, 1975). Spores of Cl. tetani have been isolated from a wide variety of other sources, including street and hospital dust, animal hair, catgut and so on.

If washed spores alone are injected into an animal they fail to germinate, are phagocytosed and do not give rise to tetanus. It has been shown that the germination of spores of Cl. tetani is dependent on the reduced oxygen tension occurring in devitalized tissue and non-viable material in the wound. Infection when it occurs remains strictly localized in the wound and the tetanic condition is due to the effects of a potent diffusible exotoxin (tetanospasmin/tetanus toxin) on the nervous system. It has been estimated that 1 kg of tetanus toxin would be sufficient to kill the population of the world (Edmondson, 1980). Tetanus toxin is one of the most toxic substances known; the estimated lethal dose for man is 60 ng (reviewed by van Heyningen, 1971). It should be noted that, unlike diphtheria, the natural disease of tetanus does not confer immunity (Vakil et al. 1964). This is because a lethal dose of tetanus toxin is too small an amount of antigen to give rise to any immunity.

#### A.4.3 Tetanus Toxin - Mode of Action

Tetanus toxin is a simple protein. Its molecular structure and characteristics together with its inactivated form (tetanus toxoid) is described in detail in Chapter 3. However, comparatively little is known about its action at the molecular level. This is in contrast

with, for example, diphtheria and cholera toxins which are quite well understood (Van Heyningen, 1971; Van Heyningen, 1980; Bizzini, 1981).

Tetanus toxin is produced by the invading bacteria at the site of infections but its effects are widespread throughout the body, although only the nervous system is directly affected. This implies immediately that there must be some transport to the site of action. The mechanism of this transport has been reviewed in detail (Bizzini, 1979, and Van Heyningen, 1981).

Most human tetanus falls into one of two categories: "general" tetanus, which is characterized by general convulsion all over the body following on early spasticity, first in the jaw muscles (lockjaw) and then moving towards the trunk and outer limbs, and "local" tetanus in which the muscles of one specific infected limb become spastic. Intramuscular injection of toxin into a limb produces local tetanus, but intravenous injection produces general tetanus. It is now thought that the toxin reaches its site of action by uptake into terminals at the neuromuscular junction followed by axonal transport in the motorneurons (Van Heyningen, 1980).

#### A.4.4 Portal of Entry

##### A.4.4.1 Accidental Tetanus

In countless situations the tetanus bacilli penetrate the human body through a break in the skin (Veronesi, 1981). The presence of foreign 'bodies' in wounds constitutes an important predisposing factor. Other lesions favouring tetanus infection are burns, skin and mucosal ulcerations, infected dental decay, bites or scratches by dogs or other animals, perforation of the earlobe and nasal septum to insert

rings or pieces of wood (Indian practices).

#### A.4.4.2 Neonatal Tetanus

Neonatal tetanus is usually a very severe disease with high lethality. The portal of entry is the umbilical cord, which was contaminated at its distal end by local application of tetanogenic substances such as earth, tobacco, coffee powder, spider webs, etc. In Africa some tribes use extracts of green bananas to dress the umbilical stump. It has been reported that 10% of 319 cases of tetanus in Bombay, India, occurred in infants born in hospital, suggesting that the instruments used to cut the umbilical cord were not adequately sterilized. In Brazil 91.6% of the mummified umbilical stumps examined were positive for tetanus bacilli cultures (Athavale, 1965); the number of cases was not mentioned.

#### A.4.4.3 Gynaecological and Obstetrical Tetanus

Most cases are a consequence of infection and necrosis of the uterus during the post-delivery period or, more frequently, after induced abortion due to septic manipulation. This form of tetanus is frequently associated with gangrene of the uterus, severe sepsis and high lethality (Veronesi, 1981).

#### A.4.4.4 Otogenic Tetanus

The usual portal of entry is the middle ear where an acute or chronic infection is present. There have been reports on the importance of otogenic tetanus among 25-30% of cases in India. This high prevalence has been related to lack of hygiene and negligence in handling middle ear otitis in India (Shah, 1956, Patel, 1965 and Lyons, 1981).



#### A.4.4.5 Idiopathic Tetanus

There are cases in which the portal of entry could not be determined precisely. Patel in India (1965) and Veronesi (1981) refer to 17% and 15.9% (respectively) of their cases where the tetanus focus could not be found (cryptogenic).

#### A.4.4.6 Other Portals of Entry

Tetanus may occur after surgery, after infection with inadequately sterilized needles (especially among drug addicts), smallpox vaccine, infected varicose ulcers, etc. (Veronesi, 1981). There has been a report of an uncommon case of tetanus which occurred after allogeneic bone marrow transplantation (Kendra, 1982).

Although tetanus is more likely to develop in untreated patients with severe badly soiled wounds than in those with clean superficial injuries, today it most commonly follows mild injuries (Willis, 1981). This is due to the protective measures that are instituted routinely in cases of severe wounding, but are so frequently omitted in cases of minor trauma. A slight penetrating wound produced by a splinter of wood or even a dirty abrasion is the type of lesion from which tetanus commonly develops.

In general, although tetanus is not seen as an epidemic and there is much under-reporting, a large number of cases are observed annually on a world-wide basis.

#### A.4.5 World Distribution of Tetanus

In general terms it may be said that the incidence of tetanus and the form that the disease is likely to take are related to the social environment and habits of a population. For example, neonatal

tetanus and puerperal tetanus are common in underdeveloped communities where standards of living, sanitation and hygiene are low. Even in more advanced societies, where tetanus is relatively uncommon, the incidence of the disease is related to such factors as military commitments, agricultural activity and drug addiction on the one hand, and by the standard and availability of wound treatment and the immune status of the population on the other.

Tetanus is a killer disease; the world wide mortality rate has been estimated at one million per annum (Bytchenko, 1975). The tetanus incidence is 1 in 25,000 of the population (Jones, 1981), and not 1 in 3 million as previously reported (DHSS 1970-76). Some 8% of the deaths in developing countries are due to tetanus in the new born (Cvjetanovic, 1981).

There are differences in mortality due to tetanus between countries at different stages of socio-economic development, with different types of agricultural practices and quality of soil. Fig.2 illustrates the world distribution of tetanus neonatorum.

#### A.4.5.1 Africa

For the period of 1971-1980 deaths from tetanus were reported irregularly from hospitals of 46 African countries. In 1979 the incidence of tetanus was known to WHO in only 27 countries. The average number of reported deaths from hospitals varied from 3000-6000 per year. It has been estimated (Bytchenko, 1981) that the mortality rates from tetanus neonatorum per 100,000 live births in 1973 were high in Burundi (3370), Benin (2990), Kenya (1030), Malawi (1090), Senegal (2970), Central African Republic (2960), Chad (2410), Gambia (1280),

Guinea (2780), Ethiopia (2720), Uganda (1120) and Zaire (1000). In Nigeria, for example, the private clinics run either by midwives or herbalists did not undertake immunization against preventable disease (including tetanus), because vaccines are usually only supplied to Government health institutions. Furthermore, some women believed that native medicine could protect against tetanus (Odumosu, 1982).

#### A.4.5.2 Asia

Deaths from tetanus were reported from only 29 countries in Asia. The available data show a marked decline in the annual incidence of tetanus in Japan and Mongolia, and rather uncertain situation in other countries. For 1973 the estimated mortality rate due to tetanus neonatorum were Nepal (3305), Bangladesh (3200), Maldives (3060), India (2754), Indonesia (1270), Pakistan (1148), Sri Lanka (448), Philippines (325), Malaysia (237), Thailand (221) and Jordan (143).

Although further studies on tetanus are necessary to have more accurate information on the true epidemiological situation, the data (Bytchenko, 1981) collected from various sources give an impression that the incidence of tetanus neonatorum in the majority of Asian countries during the period 1971-1980 was gradually declining. This trend could be explained on the basis of socio-economic changes which have taken place for the last 20 years.

#### A.4.5.3 America

Although the incidence of tetanus has decreased dramatically with the routine use of tetanus vaccine, nevertheless in 1980 alone, 95 tetanus cases were reported from 33 states (Mortality and Morbidity Weekly Report, 1981). The risk of tetanus is not limited to major

wounds; one third of patients who contract the disease in the United States have either no obvious wound or a wound considered to be trivial by the patient. The case-fatality rate of 45-55% has not changed since 1975 (Brand et al. 1983). The majority of deaths have occurred in adults over 50 years of age, either because they have never been fully immunized or because they have not received the necessary booster doses of tetanus toxoid.

In the United States the incidence is highest in the southern states such as Louisiana and Alabama, followed by Texas, Florida, Tennessee, Georgia, Missouri, Kentucky, Arizona and Arkansas. The incidence in New York City and in Washington D.C. is also high because of the number of heroin addicts (Smith, 1975).

The mortality from tetanus neonatorum was reported as 12,000 for Latin America; the case fatality rate ranging from 60-93% in different countries.

#### A.4.5.4 Europe

The distribution of the rates reveals an increase from the north to the south. The morbidity and mortality due to tetanus in Scandinavian countries and in the north of the European part of the USSR were always from 10 to 44 times lower than in the southern European countries. Since 1921 the loss of human lives from tetanus in Europe has exceeded the figure of 30,000 per 10 years. During the Second World War the incidence of tetanus increased in many European countries. After the war there was a steady decline of mortality rates along with the improvement of the standard of living. Active immunization of children and grown-ups speeded up this trend. For the first time in its history, Europe lost less than 10,000 people from tetanus during the 10-year period (1971-1980). High proportions of

new-born children among tetanus patients were reported from Greece (57%), Portugal (56%), Spain (41%), Yugoslavia (5%) and Italy (5%) (see Bytchenko, 1981). In France, tetanus accounts for 200-300 deaths per annum. It affects mostly females, and mostly aged 40-50 years (Ajjan, 1981). In a country such as Switzerland where one could expect a high percentage of vaccination against tetanus, a recent investigation carried out by 111 general practitioners showed that only 42% of urban, 30% of suburban and 28% of rural female population were adequately vaccinated (Eckermann, 1981).

In the United Kingdom, there were 19 notifications of tetanus in England and Wales in 1969 and 18 in 1980, with an increasing proportion of inpatients aged 45 and over, and a declining proportion under 15 (PHLS Public Health Laboratories Report, 1982). Limited information was available about the circumstances relating to deaths, but minor garden injuries were important in the elderly, and sports injuries in the 14-44 age group.

Recent trends of tetanus indicate that no area in the world is free from this major lethal infection. It seems that vaccination alone cannot be relied on in controlling the disease and the case fatality can be greatly reduced by modern methods of treatment.

#### A.4.6 Tetanus Prophylaxis

Different prophylactic procedures may be adopted according to the age, previous immune status of the individual and the local incidence of tetanus. The most important prophylactic procedures for the prevention of the tetanus are as follows:

#### A.4.6.1 Active Immunization

Active immunization is the prophylactic measure of choice as this establishes basal immunity before exposure to the risk of tetanus. Vaccination against tetanus forms an essential part of any routine immunization program for children. It may be instituted at any age by the administration (i.m.) of two doses of adsorbed tetanus toxoid at an interval 4-6 weeks followed by a third dose 6-12 months later. Thereafter reinforcing doses should be given every 5-10 years. Additionally, an immune patient with a tetanus prone wound should receive tetanus toxoid if the last booster was given more than 12 months previously (PHLS, Public Health Laboratory, 1982).

Since its development, in 1923, by Ramon, tetanus toxoid has been submitted to various improvements with regard to the mode of detoxification of the toxin (Gottlieb, 1967), purification, concentration and improved antigenicity (discussed in Chapter 3). In addition, tetanus toxoid, for a long time, has been combined advantageously with other antigens, such as diphtheria-pertussis (DTP, triple vaccine), typhoid and paratyphoid and more recently, with live attenuated measles vaccine (Veronesi, 1981). Also, it has been shown in animals and in man that active immunization against tetanus may be achieved with just a single shot of special tetanus toxoid (Dastur, 1981).

It is assumed that once an individual is immunized with tetanus toxoid, the circulating antibody titre will never fall to zero but will rather fall to some lower limit and remain at that level throughout the lifetime of the individual (Gottlieb, 1967). More recently, it has been questioned whether the protective blood level of antitoxin (0.01 I.U./ml) is a really safe level, since not infrequently, in some areas

of the world, individuals with blood tetanus antibody ranging from 0.01 to 1.0 I.U./ml may get tetanus, usually of mild severity and low fatality rate (Iran, Salimpour, 1978).

A long-term program of routine immunization of pregnant women with two doses of tetanus toxoid is at present being recommended by the WHO's Expanded Program on Immunization (1980) as an effective approach to the control of neonatal tetanus. The last dose is administered 2-4 weeks before delivery. Active maternal immunity is passively transferred to the fetus through the placenta. The newborn retains these maternal passive antibodies for a period of about 1 or 2 months, which is long enough to protect against eventual tetanus infection originating in the umbilical cord. For infants born to women with inadequate tetanus immunization, prophylactic treatment at birth with homologous or heterologous antitetanic sera should be considered.

#### A.4.6.1.1 Adverse Reactions to Tetanus Toxoid

Allergic reactions to tetanus toxoid occur in about 1% of individuals, mainly those who have been over-immunized (Willis, 1981). Reactions are usually local (local redness, swelling, pain), but more severe reactions such as fever, vomiting, anorexia, persistent crying and seizure have been reported (Cody et al. 1981 and Hirtz et al. 1983) in children age 0-7 years old following the immunization with DT or DTP vaccine. In March, 1979, the U.S.A. Department of Health reported four sudden deaths which had occurred since November 1978 in infants who had been vaccinated during the 24 hour period prior to death (Bernier et al. 1982).

The reactions are believed to be due to impurities of tetanus toxoid and the adjuvant (AlPO<sub>4</sub>) present in adsorbed tetanus toxoid

(Collier, 1979). However, the majority of toxoid reactors are found to have high blood levels of antitoxin (Levine et al. 1981). In order to minimize these reactions, it is recommended that plain and not adsorbed vaccine be used when reinforcement of immunity to tetanus alone is desired (Collier, 1979), and to avoid hyperimmunization.

Active immunization does not provide any protection in a non-immune patient who requires immediate prophylaxis (Willis, 1981).

#### A.4.6.2 Passive Immunization

Generally speaking, administration of antitoxin to the non-immunized is indicated for all people with a soiled wound. Sufficient amounts of tetanus antitoxin should be inoculated as soon as possible in an attempt to block the toxin from being fixed by the central nervous system (CNS). It is much more difficult to neutralize the toxin which has already been fixed by the nervous structures (gangliosides). As a consequence of this, passive immunization should be given very shortly after injury, before any toxin can reach its target cells in the CNS.

It has been shown experimentally that large doses (1000 IU/kg) of antitetanus serum (ATS) may prevent tetanus in guinea-pigs if given not later than 6 hours after the inoculation of a lethal dose of tetanus toxin (Bytchenko et al. 1970).

##### A.4.6.2.1 Heterologous Passive Immunization

The use of heterologous ATS, usually equine or bovine, is based on the property of the tetanus antitoxin to neutralize fully the tetanus toxin, provided an adequate amount of ATS is added in vitro to the toxin. However, when the ATS is challenged in vivo against the toxin, a



greater amount of ATS is required to produce similar results (Veronesi, 1981). The most common dosage being used is 1500-3000 I.U. (i.m.).

The chief disadvantages of heterologous antitetanus serum are allergic reactions (more on this later) which are likely to follow its use, and the accelerated elimination of antiserum from sensitized patients. The latter is of some importance, since in these patients quite substantial doses of antitoxin provide inadequate protection. Patients must be tested for sensitivity to heterologous antitoxin before a full dose is administered. Intradermal testing is unreliable, and the trial dose method is used. This involves subcutaneous injection of 0.1 ml of the serum and the patient's general condition is observed before a larger dose is given. If the patient is hypersensitive further heterologous serum must not be given (Willis, 1981).

#### A.4.6.2.2 Tetanus Immunoglobulin of Human Origin (TIG)

The need for homologous instead of heterologous ATS became more obvious when the harmful effects of heterologous ATS were followed by the failure that such prophylaxis showed during World War II (Eckmann, 1963).

Homologous antitoxin provides better protection and has none of the disadvantages of animal antiserum. Hypersensitivity reactions to human immunoglobulin are rare (if injected i.m.), and there is no risk of its accelerated elimination. A single dose of 250-500 I.U. provides protection for about 4 weeks (Willis, 1981).

The usual prophylactic dose of human TIG is 250 units for tetanus from wounds without adequate, prior, tetanus toxoid immunization or in the event of immunological problems. In the event of severe,

neglected or old (more than 24 hours) wounds, 500 units may be administered (Furste et al. 1981). The available preparations are formulated for intramuscular use only, since intravenous injection of TIG has caused reactions (Janeway, 1970).

Passive immunization with fragments of tetanus antitoxin was introduced in the hope of overcoming several disadvantages of ATS. First of all it would activate complement less. Secondly, it was hoped that the fragments would distribute better than IgG into tissue spaces or even into cells because of their lower molecular weight. Thirdly, it was hoped that fragments made from heterologous IgG would be less immunogenic than the parent IgG, and that consequently their rate of immune elimination and their ability to induce unwanted immune reactions would be lower.

Two types of fragments, Fab (Bizzini, 1981) and F(ab)<sub>2</sub> (Erdman, 1981) were investigated. Fab is produced by papain digestion and F(ab)<sub>2</sub> is produced by pepsin digestion of the Ig molecules. Fab soon turned out to be less suitable for therapeutic purposes. Its neutralizing potency is low, and in animal experiments, the speed of elimination was very high. Fab preparations have not been used for tetanus prevention in man. Homologous F(ab)<sub>2</sub> is marketed for passive immunization in tetanus. Its neutralizing potency is higher in comparison with Fab, but still lower (about 50%) in comparison with IgG. Heterologous F(ab)<sub>2</sub> is as immunogenic as its parent IgG, and is eliminated faster (Wellhöner, 1981).

#### A.4.6.3 Active-Passive Immunization

Active-passive immunization is advised for those who have never been vaccinated against tetanus and are in need of optimal protection against this disease. First a dose of adsorbed tetanus toxoid is given to the person at risk who would also receive tetanus antitoxin (homologous or heterologous). The principle is that when passive immunity is declining, active immunity appears and thus avoids the unprotected period when tetanus toxin could cause tetanus. Naturally to avoid interference between the toxoid and the antitoxin, the products should be injected separately, using a different syringe and injection site. The patient will have to return later for the rest of the course of active immunization. The first dose of toxoid does not provide any protection in non-immune patients who require immediate prophylaxis (PHLS, Public Health Laboratory, 1982).

#### A.4.6.4 Antimicrobial Prophylaxis

Although the prophylactic administration of antibiotics to all cases of open wounds is not recommended, it has been suggested that antimicrobial prophylaxis, combined with surgical treatment of wounds, should replace heterologous tetanus antitoxin (Veronesi, 1981). The efficacy of antimicrobial prophylaxis is dependent not only on the sensitivity of contaminating strains to the drug, but also on the active concentration of the drug attainable at the site of contamination. Most strains of C1. tetani are highly sensitive to penicillin, metronidazole, tetracycline and erythromycin. In order to be effective, however, prophylaxis must be commenced soon after injury, within 6 hours, and continued for at least 5 days. Antimicrobial agents are not active against tetanus spores and have no effect upon preformed toxin (Willis, 1981).

Although the value of antimicrobials as a replacement for antisera remains unproven, chemoprophylaxis, used with care and discretion, could play an important part in tetanus prevention, especially when antitoxin is not available.

#### A.4.6.5 Surgical Prophylaxis

Prompt and adequate wound toilet and proper surgical debridement of wounds are of paramount importance in the prevention of tetanus as there is an increased risk that tetanus spores may germinate in a wound if there is delay in cleansing. Surgery is essential for all wounds, no matter how trivial. It may range from medical excision to simple washing; the aim is to remove dirt, foreign bodies, necrotic tissue, and blood clots, and to restore the blood supply to the part. Antimicrobials may be administered systemically before surgery and may be applied topically as part of the surgical toilet (PHLS, Public Health Laboratory, 1981). Of course ATS when indicated will ensure safer surgical prophylaxis.

Wound management including tetanus prophylaxis schedule employed by Lothian Health Board is outlined in Table 5.

#### A.4.7 Treatment of Tetanus

The treatment of established tetanus has four aspects: (1) prevention of absorption of further toxin by the administration of antitoxin, and by surgery of the wound, (2) control of reflex spasms, (3) prevention of intercurrent pulmonary or other infection, (4) control of fluid and electrolyte balance, and maintenance of nutrition. Many accounts of treatment have been published (see for example Edmondson, 1980; Ray et al. 1981; Willis 1981, Duguid et al., 1978 and Sanders 1979).

As previously stressed tetanus is particularly widespread at the present in the developing and underdeveloped countries. In these less fortunate countries, hospitals are very modestly equipped. A specialized unit serving 1 million inhabitants may have to admit up to 500 cases of tetanus per year (Ray et al. 1981). Intensive care is therefore completely inaccessible to the vast majority of patients who develop tetanus throughout the world, thus justifying the need for simplified and effective treatment of the disease by such means as serum therapy.

#### A.4.7.1 Serum Therapy

In the treatment of established tetanus, antitoxin is of proven value. Heterologous ATS has long been considered the principal element in the curative treatment of the disease. It is often administered in very large doses, e.g. up to 1 million units (Ray et al. 1981) by all possible routes; in the neighbourhood of the wound, intramuscularly, subcutaneously, intravenously, intrathecally and in association if necessary.

The value of ATS has been questioned over the last 20 years, mainly because of the risk of adverse reactions, particularly "serum sickness" which although not very dangerous, is common (46% of cases). In experimental studies, in which it was decided to omit serum therapy completely, a significant increase in mortality was observed and caused the trial to be discontinued (Patel et al. 1967).

As a precaution, even if most authors have abandoned massive serum therapy, they still give moderate doses of ATS, such as 3,000 and 6,000 I.U. generally by the intramuscular route (Veronesi, (1981). A preliminary intradermal test is advised to avoid anaphylactic reactions. Comparison of the efficacy of different doses of tetanus antitoxin has been carried out by Vakili et al. (1963 and 1968).

An additional argument in favour of not abandoning serum therapy was provided by Patel's demonstration (1967) of circulating toxin in the blood, in 10% of cases, and in the cerebrospinal fluid (CSF) in 4% at the outset of the disease.

Interest in passive immunotherapy has been rekindled as a result of the introduction of human specific gamma-globulin, which has the advantages of avoiding the risk of adverse reactions, if not injected intravenously (Janeway, 1970), and with the return to favour of the intrathecal route, recommended by Ildirim (1970).

#### A.4.7.2 Intrathecal Injection of Tetanus Antitoxin

As long ago as 1898, Roux and Borel injected tetanus antitoxin (Behring's) into different parts of the central nervous system (CNS) of guinea-pigs. From their results, they suggested a direct action of tetanus toxin on the CNS.

Sherrington (1917) and Smith (1965) have also demonstrated a beneficial effect of antitoxin injection directly into the CNS. After intrathecal injection of ATS, animals have been saved at the stage when neither intramuscular nor intravenous injections of antitoxin produced any favourable response. However, at the later stage of tetanus intrathecal antitoxin was not effective.

Although the intrathecal route was later abandoned because of dangerous side-effects, interest has been rekindled by observations of Ildirim (1975), first in the dog and then in the newborn infant. With no intensive care, the mortality from neonatal tetanus has been reduced from 90 to 17% by the intraspinal administration of 2500 I.U. of tetanus antitoxin or of 250 I.U. of TIG, together with 10,000 I.U. injected by the pre-umbilical, intramuscular or intravenous route.

Encouraging results (with the exception of Vakil, 1981) have been reported more recently by Gupta et al. (1980), Singh et al. (1980) and Thomas et al. (1982). The latter has used a combination of ATS and steroid, first used by Sanders et al. (1977).

Intrathecal serum therapy which would appear to be a logical course of action, is indeed almost certainly of some effectiveness, and is being employed by an increasing number of physicians. The optimal doses are 30 units per kg in adults, and 45 units per kg in children (Ray et al. 1981). This route not only seems to be a more effective route, it also requires much lower dose of antitoxin than that given systemically (Smith, 1965). This finding is related to the barrier which exists to the passage of antitoxin from the circulation into the brain. With the availability of more specific and purer TIG (human) and removal of one major concern over safety, it is beginning to look as though there could be a role for the intrathecal route in the treatment of tetanus.

#### A.5 Limitations of Conventional Methods

The most widely used immunoglobulin preparations have been those prepared by modifications of the ethanol fractionation procedure of Cohn. In the mid-1940's, Janeway and Berenberg documented that the intravenous injection of immunoglobulin derived from Cohn fraction II caused marked adverse reactions. The severity of these reactions prompted the warning that the preparations were to be used intramuscularly only. Efforts to eliminate the adverse reaction by further purification of the immunoglobulin and attempts to develop an adequate screening test in animals for detection of reactive preparations all ended in failure (Janeway, 1970). After 40 years the

problems still remain; indeed even intramuscular administration of these preparations has been found to cause reactions, ranging from pain at the injection site to systemic effects (Finlayson, 1980). The incidence is especially high (20%) in patients with antibody deficiency receiving regular injections (Thompson, 1981).

Some of the reasons for these reactions have been (a) aggregated IgG, (b) preservatives and (c) impurities present in the preparations. The amount of aggregates vary from 0.1-3%. Functionally these aggregates will activate complement, and spontaneous complement activation by infused Ig-preparations may lead to severe reactions. These aggregates are formed during the plasma fractionation process, such as the cold ethanol or ammonium sulphate procedures (Römer et al. 1981). Immunoglobulin preparations obtained by these methods have to be further processed in order to obtain good intravenous tolerance in high-risk individuals.

Some preparations use mercury based preservatives to maintain sterility. This metal accumulates, and has been implicated as a cause of diarrhoea in some patients. However, this is considered uncertain (Thompson, 1981).

Impurities such as vasoactive enzyme prekallikrein activator (PKA) and kallikrein or plasmin can be present in some preparations. When preparations containing these enzymic contaminants are given intravenously, reactions could include flushing, dyspnoea and tightness in the chest (Alving, 1980). These preparations have been tolerated better if diluted and slowly transfused (Welch et al. 1980).

In the case of an unexplained adverse reaction to an immunoglobulin preparations, recipients have sometimes been investigated for antibodies to IgG. In some cases, hypersensitivity



reactions result from previous sensitization of the recipient to special antigens, particularly IgA, or other immunogenic substances present in the IgG preparation. Antibodies to IgA may be found in individuals with a selective IgG deficiency, who have previously been treated with immunoglobulins for recurrent infections or given blood or plasma transfusion for other reasons. In such patients the administration of blood and plasma is strictly contra-indicated (Barandun et al. 1981).

In the past few years, several new immunoglobulin preparations for intravenous application have been developed. The earliest manufacturing approach was to isolate the immunoglobulin by a conventional procedure, and then to modify the isolated protein. Modifying agents included the enzymes pepsin (Schultz et al. 1962), which had long been used in the processing of equine antitoxins, and plasmin (Sgouris, 1967); both were used to remove the Fc-part of IgG. Chemical modification of the IgG molecule was done by  $\beta$ -propiolactone (Stephan, 1975), by treatment with hydrochloric acid at pH4 (Barandun et al. 1962), or by reductants for cleaving disulphide bridges (Masuho et al. 1977).

More recently, producers have attempted to modify the fractionation procedure itself, so as to obtain immunoglobulins suitable for intravenous use without enzymic or chemical treatment. The methods developed include precipitation procedures, ion exchange techniques, or combinations of both (Welsh et al. 1980; Eibl, 1980 and Hoppe et al. 1973).

The introduction of immunoglobulin preparations which can be given in high intravenous doses has been accompanied by the observation of side-effects, as described earlier. The various forms of intolerance

were classified according to their aetiology as discussed in the most recent workshop in immunotherapy (Barandun et al. 1981). Therefore only intramuscular injections were once again recommended before safe preparations for intravenous infusion were available (Hitzig, 1981). Although intramuscular (i.m.) injections may be easier to apply, in all other respects the intravenous (i.v.) application is superior, in particular for the following three points: (a) because of the large volumes often needed, i.m. injection is difficult and must be distributed to several sites, (b) i.m. injection is painful for several hours or days, (c) injected immunoglobulin is partly destroyed (40-60%) by proteolytic enzymes (Hitzig, 1981). In this connection, it should be noted that others have recently claimed that certain immunoglobulin preparations can be safely given by the i.v. route (Yap et al. 1983).

The above problems could be solved by developing techniques to produce potent and specific antibody preparations against particular viruses or bacteria (which is the subject of this thesis), these preparations having the two virtues of not only high and reproducible antibody titres against specific organism, but also containing no impurities which are the main cause of adverse reactions. Therefore low doses of a preparation containing high level of antibody directed only against the relevant pathogen can be administered with safety.

The most attractive ways to obtain such preparations would be by using the natural specificity of antigen-antibody reactions as in affinity chromatography technique, or by establishing a cell line capable of producing the monoclonal antibody of our choice suitable for human use. Both approaches were used to produce specific anti-tetanus antibody, and are explained in detail in Chapters 5 and 6 respectively.

#### A.5.1 Limitations of Available Tetanus Antitoxin Preparations

Animal anti-tetanus serum (ATS as referred to earlier) is a heterologous protein to man and is identified as a strong antigen by the human immune system. The unwanted reactions are both numerous and dangerous (Moynihan, 1955; Binns, 1961, Skudder et al. 1962 and Cox et al. 1963). The incidence of unwanted reactions is between 5 and 10% or even higher. The most dangerous reaction is anaphylactic shock which may be evoked even by a small dose used in the intradermal skin test (Buff, 1960). The first ATS injection may evoke the production of anti-horse IgG antibodies. These antibodies form immune complexes with ATS which lead to complement activation (as described earlier). A third group of side-effects comprises serum disease (described for ATS as early as 1938 by Lyall et al.). These result from the formation and deposition on the vessel walls of immune complexes (composed of ATS and precipitating IgG) with subsequent complement activation. Heterologous ATS has been rendered less prone to produce hypersensitivity reactions by new methods of purification that may remove most of the non-specific immunoglobulin and other proteins responsible for such reactions (Robb, 1975 and Tardy et al. 1978). In spite of these advances, anaphylactic reactions may still occur in individuals receiving heterologous ATS (Veronesi, 1981).

The need for homologous instead of heterologous ATS is obvious. In this context, Rubbo (1967) said "There is a growing awareness that this agent (ATS) is a potentially dangerous allergen and that a charge of negligence could be brought against a doctor, not because it was withheld but because it was used".

The use of human TIG has gradually increased, mainly in developed areas where tetanus is already under control. In some highly developed

countries, more deaths have been caused from anaphylactic shock due to heterologous ATS than from tetanus itself (Ericksson et al. 1967).

The advantages of TIG (H) over heterologous ATS are (a) duration of immunity is longer than that obtained with heterologous ATS, (b) lower doses of homologous antitoxin (TIG (H)) are highly effective, (c) dramatic reduction in side-effects. However, a few failures have also been accredited to TIG (H) (see Tetanus Surveillance Report, 1974) since the preparation of TIG (H) is identical to that of human serum globulin (Cohn fraction II) except that hyperimmunized donors are used. This preparation still contains large amounts of other non-specific antibodies and other impurities. As a result similar side-effects to those produced by using other IgG preparations (Cohn II fraction) are observed (see A.5.1). Furthermore, TIG (H), like other IgG preparations, is restricted to intramuscular administration.

#### A.6 Aims of the Current Project

When these studies commenced in 1979 the overall objective was to develop novel procedures for producing an anti-tetanus antibody rich preparation which would be used therapeutically. The present thesis deals with investigations which had to be performed in the pursuance of this objective; these studies are as follows.

In the initial phase sensitive ELISA assays had to be developed which would permit the measurement of circulating anti-tetanus antibodies in both man and mouse and in antibody preparations derived from the plasma or serum thereof. Furthermore, in view of the fact that I also planned to attempt to purify anti-tetanus antibodies by immuno-affinity procedures, it was deemed necessary to develop assays capable of measuring the affinity of such antibodies.

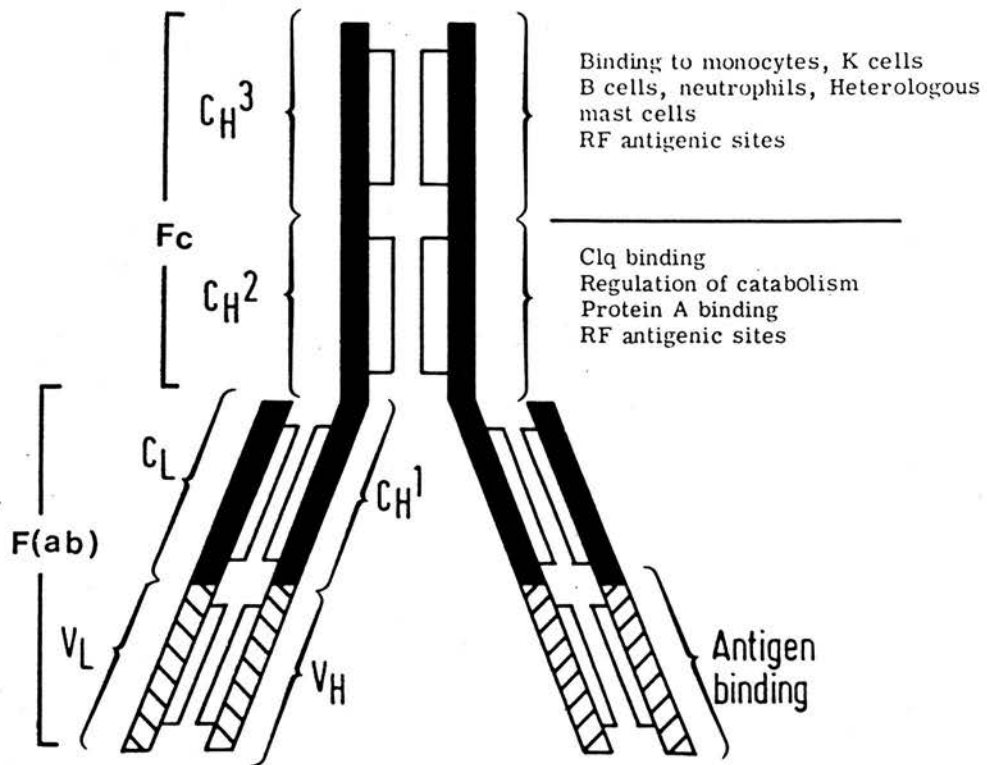
While our prime interest was in the antibodies themselves, it was felt in the light of the studies we propose to do that I ought to investigate the heterogeneity of various tetanus-toxoid preparations. This seemed particularly important in view of the apparent lack of information in this area.

From the outset it was clear that the two most obvious ways to obtain enriched anti-tetanus antibody preparations were affinity chromatography of polyclonal antibody preparations or by the monoclonal antibody technique. Both these approaches have been attempted in the present study. However before attempting either approach, a certain amount of additional background work was necessary.

In the case of the immuno-affinity studies where I was attempting to enrich antibodies from polyclonal antibody preparations, a number of studies had to be undertaken to establish the optimum conditions for the preparation of immunoadsorbents and to evaluate various factors which might influence their performance. Finally before attempting to produce murine monoclonal antibodies to tetanus toxoid we felt it advisable to investigate in detail the immune response of mice to different tetanus toxoid preparations paying specific attention to the isotype of the antibody elicited. The reason for performing all these investigations is explained in greater detail in the thesis.

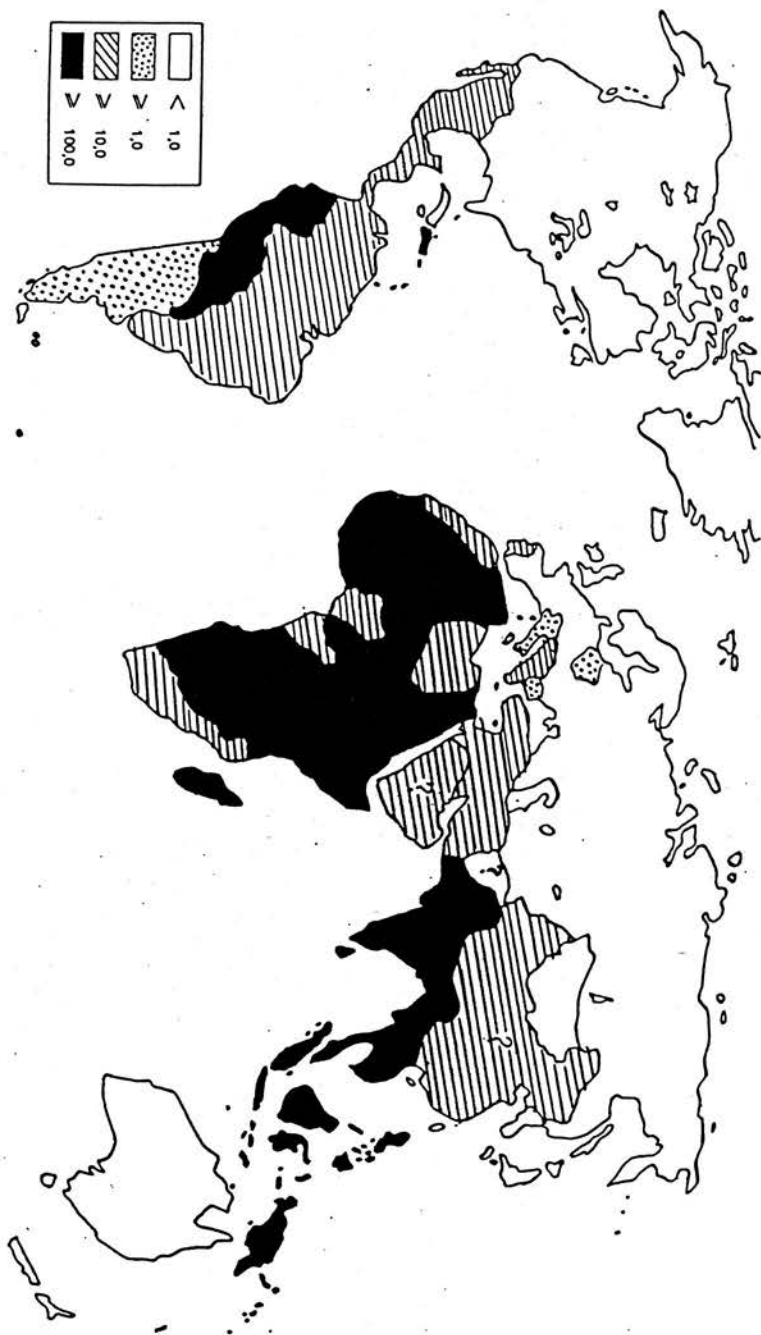
FIG. A-1

ACTIVITIES ASSOCIATED WITH VARIOUS DOMAINS OF IgG



Adapted from a figure kindly provided by Dr. K. James.

*Fig.A-2 World distribution of tetanus neonatorum.  
Expected mortality rates per 100 000 live births (1973)*



Adapted from Bytchenko (1981), produced in 6th International Conference on Tetanus in Lyon, France.

TABLE A-1

APPLICATIONS OF STANDARD HUMAN SERUM GLOBULIN (HISG)\*

Disease	Principal Use	Reference
Hypogammaglobulinaemia or Ab-immunodeficiencies	Treatment	Buckley, 1980 Asherson, 1980 Cunningham <u>et al.</u> 1981 McClelland <u>et al.</u> 1984 (review) Stiehm, 1979 (review)
Hepatitis A	Prevention	Stokes <u>et al.</u> 1945 Suff <u>et al.</u> 1979
Hepatitis non-A non B	Prevention	Zuckerman, 1979 (review)
Measles	Prevention	Stokes <u>et al.</u> 1944 Stiehm, 1980
Rubella	Prevention	Schiff, 1969 Stiehm, 1979 (review)
Poliomyelitis	Prevention (efficacy doubtful)	Hammon <u>et al.</u> (1954) Stiehm, 1979 (review)

\*HISG is prepared by alcohol fractionation of pooled human serum by the Cohn procedure (Cohn fraction II). It is for intramuscular use only, and is available in 2-10 ml vials from several manufacturers



TABLE A-2

## SPECIFIC SERUM GLOBULINS AVAILABLE FOR PASSIVE IMMUNITY\*

Disease		Principle Use	Reference
Hepatitis B	HBIG	Prevention	Reesnik et al. 1979 Tabor, 1980 Stiehm, 1979b (review)
Varicella Zoster	VZIG, ZIG	Prevention	Zala et al. 1980 and 1983 Stiehm, 1979 (review)
Vaccinia	VIG	Prevention and treatment	Stiehm, 1979 (review) McClelland et al. 1984 (review)
Pertussis		Treatment (efficacy doubtful)	Stiehm, 1979 (review)
Mumps		Prevention (efficacy doubtful)	Stiehm, 1979 (review)
Tetanus	TIG	Prevention and treatment	Veronesi, 1981 (review) Stiehm, 1979 (review)
Rabies	RIG	Prevention	Mertz, 1982 Stiehm, 1979 (review)
Rh (D) hemolytic	RhIG	Prevention	Diamond, 1970 Smith et al. 1972 McClelland et al. 1984 (review)

\*Preparation of these immunoglobulins is identical to that of HSIG (Cohn fraction II) except that donors immunized to or convalescing from a specific illness are used.

TABLE A-3

ANIMAL SERA AVAILABLE FOR PASSIVE IMMUNITY

Product	Principle Use
Diphtheria antitoxin	Prevention and treatment
Tetanus antitoxin (ATS)	Prevention and treatment (when TIG not available)
Rabies immune serum	Prevention and treatment (when RIG not available)
Botulism antiserum	Treatment
Gas gangrene antiserum	Treatment (efficacy doubtful)
Black Widow spider antivenin	Treatment of spider bite
Crotalide antivenin	Treatment of snake bite

Reference: Stiehm, 1979 (review)

TABLE A-4

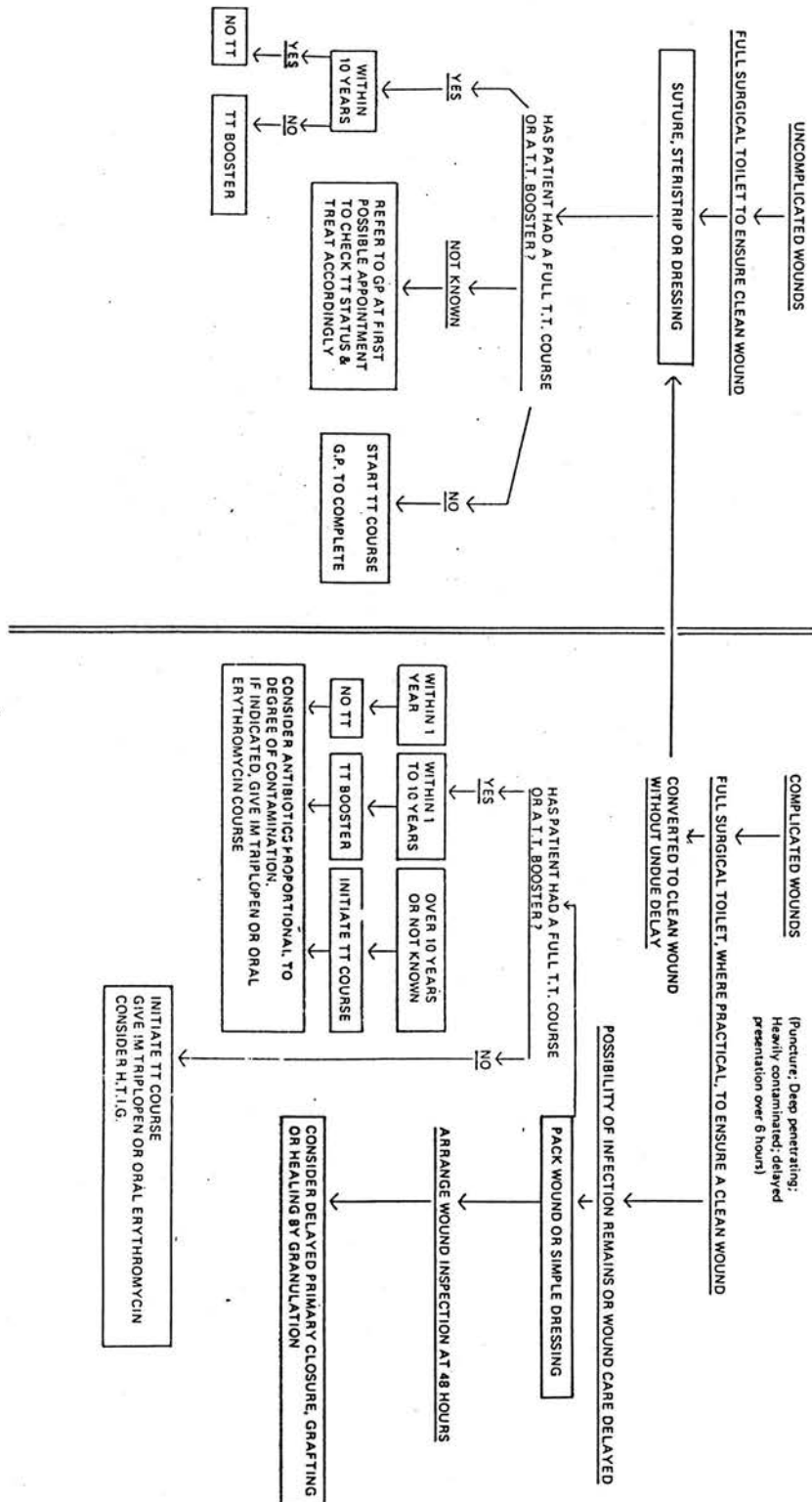
MONOCLONAL ANTIBODIES WITH POSSIBLE USE IN PASSIVE IMMUNITY\*

Micro-organism	Origin	Reference
<u>Clostridium tetani</u>	Human	Gigiliotti <u>et al.</u> 1982 Boyd <u>et al.</u> 1982
<u>Haemophilus influenza</u> <u>Type B</u>	Human Rat	Hunter <u>et al.</u> 1982 Hason <u>et al.</u> 1982
<u>Escherichia coli</u>	Mouse	Söderstrom <u>et al.</u> 1983 Young, 1983
<u>Group B Streptococci</u>	Mouse	Harris <u>et al.</u> 1982 Thomas <u>et al.</u> 1982
<u>Pseudomonas aruginosa</u>	Mouse	McClelland, 1984
<u>Herpes Virus</u>	Mouse	McMichael <u>et al.</u> 1981
<u>Protozoa (Malaria)</u>	Mouse	McMichael <u>et al.</u> 1981

\*Adapted from McClelland, 1984 and McMichael et al. 1981 (reviews)

TABLE A-5

WOUND MANAGEMENT INCLUDING TETANUS PROPHYLAXIS SCHEDULE



N.B. Tetanus prophylaxis measures, recommended in Accident and Emergency Department of the Royal Infirmary, Edinburgh.

T.T.: TETANUS TOXOID  
H.T.I.G.: HUMAN TETANUS IMMUNOGLOBULIN

## B. GENERAL MATERIALS AND METHODS

## B. GENERAL MATERIALS AND METHODS

### B.1 TOTAL PROTEIN ESTIMATION

Total protein concentrations were initially determined by the Folin-phenol procedure of Lowry et al. (1951). The protein standard used was crystallised bovine serum albumin (Armour Pharmaceutical Company, Phoenix, U.S.A.). A standard curve was constructed by assaying 10-12 standards (in duplicate) ranging from 25 to 1000  $\mu\text{g}$  protein per ml (Fig. 1 and Table 1). All estimations were performed in duplicate.

In later studies the protein concentration of tetanus toxoid preparations was determined by measuring the absorbance at 280 n.m. in an SP-500 spectrophotometer (Pye Unicam, Cambridge, U.K.), using an extinction coefficient ( $E_{1\text{ cm}}^{1\%}$ ) for tetanus toxoid of 4.2. This extinction coefficient was determined by measuring the O.D. of a range of tetanus toxoid preparations of known protein concentration (determined by the above Folin-phenol technique (Fig. 2 and Table 2)). The protein concentration of the toxoids was calculated using the following formula:-

$$\frac{E_{280\text{ nm}}^{1\text{ cm}} \times \text{dilution}}{4.2} = \text{Protein concentration in mg/ml}$$

### B.2 PROTEIN CONCENTRATION PROCEDURES

Two different procedures were used to concentrate samples.

#### 2.1 Ultrafiltration

A length of 8/32 "Visking" dialysis tubing (Gallenkamp, London, U.K.) was soaked for 5 min in distilled water and threaded through a rubber bung. A plastic filter funnel was then inserted into the

dialysis tubing, and a firm seal made by inserting the end of the filter funnel into the hole in the bung. The other end was tied off. The sample to be concentrated was poured into the tubing and the bung was inserted into the neck of a 250 ml Buchner flask with side arm. The flask was evacuated and left at 4°C until the sample was concentrated to the desired volume. The optical density of the filtrate and the concentrate was measured at 280 n.m. to check for leakage.

## 2.2 Minicon-B clinical sample concentrator

These were obtained from Amicon Ltd. (Woking, Surrey, U.K.). They contained eight isolated sample chambers which held up to 5 ml of sample and retain molecules with a M.W. greater than 27,000 M.W. The inner surface of the chambers is a membrane of selective permeability, backed by absorbent pads. Retained constituents are progressively concentrated in the chambers as fluid is absorbed by the pads. Samples can be concentrated 5-100 times as indicated by graduation lines.

## B.3 IODINE LABELLING OF PROTEINS

Iodine, in particular  $^{125}\text{I}$  was the isotope of choice for a number of reasons. (i) It is a  $\gamma$ -emitting isotope which is preferable to  $\beta$ -emitters such as  $^3\text{H}$  or  $^{14}\text{C}$ . The relative advantage of radioiodine may be seen by a simple calculation that one atom substituted into a molecule of insulin provides 200 times as many radioactive disintegration per unit as would be produced if all 263 carbon atoms in the molecule were  $^{14}\text{C}$ ; 600 atoms of  $^3\text{H}$  would be required to bring the same number of counts (Hunter, 1981). (ii)  $^{125}\text{I}$  has a longer half life (60 days) and higher isotopic abundance

(80-95%) in commercially available preparations than  $^{131}\text{I}$  with its shorter half life (8 days) and lower isotope abundance (15-20%).

(iii)  $^{125}\text{I}$  has a higher counting efficiency and is relatively less hazardous to work with than  $^{131}\text{I}$  because of its lower decay energy.

(iv) Although under some circumstances iodine may react with sulphhydryl groups, with histidine or with tryptophan, the chemistry of radioiodination is predominantly the substitution of iodine into tyrosine groups (Hughes, 1975). This is achieved by using a pH just on the alkaline side of neutrality. The reaction consists of substitution of iodine, produced by oxidation of iodine into ionized tyrosine (phenate) groups of the protein. Various oxidizing agents have been used.

Two methods were used to radio-label proteins, both based on the method of Greenwood et al (1962) utilising chloramine-T as oxidising agent. The difference between the two methods was the nature of the reducing agent used to neutralise the chloramine-T. In earlier studies sodium metabisulphite was used to protect the protein from oxidation damage but later this was replaced by cysteine-HCl (Brown et al. (1982).

### 3.1 Iodination using $\text{Na}_2\text{S}_2\text{O}_5$ as reducing agent

To 200-400  $\mu\text{g}$  of protein in a glass bijoux, the following reagents were added in sequence:-

- (a) 100  $\mu\text{l}$  phosphate buffer (0.5 M pH 7.6)
- (b) 10  $\mu\text{l}$  (1 mCi)  $\text{Na } ^{125}\text{I}$  (Radiochemical Centre, Amersham, U.K.)
- (c) 100  $\mu\text{l}$  (400  $\mu\text{g}$ ) chloramine-T (60-90 seconds incubation with mixing on a magnetic stirrer)
- (d) 100  $\mu\text{l}$  (800  $\mu\text{g}$ )  $\text{Na}_2\text{S}_2\text{O}_5$
- (e) 100  $\mu\text{l}$  (2 mg) sodium iodide



The mixture was immediately applied to a G-25 or G-50 Sephadex column (Pharmacia Fine Chemicals, Uppsala, Sweden). The column had been equilibrated with phosphate buffered saline pH 7.2 (PBS) and presaturated with excess bovine serum albumin (10-20 mg B.S.A. in 200  $\mu$ l) to minimise adsorption of labelled protein to the Sephadex. Bromophenol blue (1%) and phenol red (1%) with B.S.A. were first added to estimate the void volume of the column and to ensure that the column had been properly packed. Twelve 3.0 ml sample fractions were collected, and 10  $\mu$ l aliquots of each counted on an Ultragamma counter (1280 LKB, South Croydon, U.K.). To these aliquots was added 100  $\mu$ l foetal calf serum (FCS - Gibco Biocult, Paisley, U.K.) and an equal volume of trichloroacetic acid (TCA 20%). The TCA-precipitated counts were expressed as a percentage of the total count to measure the  $^{125}\text{I}$  that was protein bound. The total and TCA-precipitable counts were then plotted against the fraction number (Fig.3). The counts in each fraction were converted into  $\mu\text{Ci}$  terms using the formula:-

$$\frac{C}{T} \times \frac{VT}{VC} \times \frac{100}{58} \times \frac{1}{37,000} = \text{ } \mu\text{Ci}$$

where C = counts

VT = fraction volume in  $\mu$ l

VC = volume counted in  $\mu$ l

$\frac{100}{58}$  = the reciprocal of the counting efficiency of the gamma counter and converts counts to disintegrations

37,000 = the number of disintegrations/second per  $\mu\text{Ci}$ .

T = counting time in seconds.

The fraction(s) exhibiting highest radioactivity and TCA precipitability were chosen and stored at  $-20^{\circ}\text{C}$  until use.

### 3.2 Iodination using cysteine-HCl as reducing agent

In later experiments, in order to minimise damage to the protein resulting from oxidation, cysteine-HCl was substituted for  $\text{Na}_2\text{S}_2\text{O}_5$  and a lower concentration of choramine-T with a shorter incubation period was chosen (Brown *et al.*, 1982).

To 10-300  $\mu\text{g}$  of protein the following were added in sequence:-

- (a) 100  $\mu\text{l}$  phosphate buffer pH 7.5 (0.25M)
- (b) 10  $\mu\text{l}$   $\text{Na}^{125}\text{I}$  or  $\text{Na}^{131}\text{I}$  (10  $\mu\text{Ci}/\mu\text{g}$  protein)
- (c) 10  $\mu\text{l}$  (25  $\mu\text{g}$ ) choramine-T (15 second incubation with mixing)
- (d) 100  $\mu\text{l}$  (62  $\mu\text{g}$ ) cysteine-HCl
- (e) 855  $\mu\text{l}$  (5 mg) KI

Protein was separated from free iodine as described in section 3.1.

### 3.3 Efficiency of radio-iodine labelling procedure

Labelling efficiency may vary depending on the protein being labelled, its molecular size, the number of tyrosine group it contains, its purity and concentration. In this study the Wellcome tetanus toxoid labelled less efficiently than Connaught toxoid, IgG and BSA (Table 3).

### B.4 GEL FILTRATION

Gel filtration was performed on Sephadex (Pharmacia Fine Chemicals, Uppsala, Sweden). G-25, G-50 and G-100 (Superfine grade) were used for desalting, buffer exchange purposes and removal of free label after iodination of proteins. G-200 Sephadex was used for separation of protein mixtures (mainly tetanus-toxoid).

#### 4.1 Preparation of Sephadex gel

The dry powders were swollen in approximately 5 times their weight of 0.06 M PBS pH 7.2 containing 0.02% NaN<sub>3</sub> at room temperature for 3 days for G-200 and 3 hours for the other types of Sephadex. After swelling, the Sephadex was allowed to settle and the fines were removed by decantation. The gel was suspended in PBS and this process was repeated until the majority of fines had been removed.

#### 4.2 Packing the gel bed

A column manufactured by Wright Scientific Limited (Stonehouse, Surrey, U.K.), fitted with a sintered glass pad at one end, was partly filled with buffer to check for leaks. The column was then clamped in a vertical position, and the swollen Sephadex was poured down a glass rod into the column. Before the Sephadex had fully settled, more slurry was added and the process repeated until the column was completely full. The column was then sealed and a variable speed peristaltic pump (LKB 12000, Varioperpex) connected to the bottom of the column. The flow rate was adjusted to that appropriate for the gel type and column size used, and at least one column volume of the buffer was pumped through. The efficiency of the G-200 columns was tested before each experiment by performing a trial run with normal human serum under the same conditions to be used with the experimental samples. The elution profile of the major protein peaks were used to calibrate the column.

#### B.5 PROTEIN-A SEPHAROSE CHROMATOGRAPHY

Protein-A is extracted from the cell wall of staphylococcus aureus. It consists of a single polypeptide chain of molecular weight

of around 41,000 daltons and contains binding sites that show a high affinity for the  $F_c$  portion of immunoglobulin-G (IgG). Each protein-A molecule is able to bind two molecules of IgG. Protein-A interacts with IgG of many species and within a species this interaction may be restricted to certain subclasses (isotypes) of IgG. For example, in man, protein-A interacts more strongly with IgG of subclasses 1,2 and 4 than with IgG<sub>3</sub>, and in mouse with all IgG subclasses but not with IgM or IgA (Kronvall et al., 1970; Grey et al., 1971).

Protein-A Sepharose (Pharmacia) was used to fractionate mouse IgG subclasses. The procedure used (Ey et al., 1978) relies on the fact that the binding of mouse Ig-isotypes to Protein-A Sepharose is pH sensitive. Staphylococcal protein-A covalently linked to Sepharose CL-4B (Protein-A Sepharose) was obtained from Pharmacia Fine Chemicals. 1.5 grams were swollen in 10 m M PBS pH 8.0 containing 0.05% NaN<sub>3</sub> and packed in a 20 ml column. Before use, the column was equilibrated with 0.1 M PBS pH 8.0. Mouse serum or ascitic fluid (1 ml + 1 ml PBS) was applied to the column. The unbound immunoglobulins (M,A and E) and other serum proteins were washed off using the equilibrating buffer. The various IgG subclasses were recovered by stepwise elution with citrate buffer of decreasing pH. After each separation the Sephadex column was washed thoroughly with phosphate buffer pH 8.0 and stored at 4°C. The column was used several times without any noticeable change in its binding properties.

#### B.6 IMMUNO-AFFINITY CHROMATOGRAPHY

This is a form of adsorption chromatography in which the bed material has immunologically specific affinity for the substance to be

isolated. The specific adsorptive properties of the bed material (CNBr-activated Sepharose-4B) are obtained by covalently coupling a relevant molecule (antigen or antibody) to the insoluble matrix. The complementary antibody or antigen is isolated from the starting material by passing it through the column, the specially adsorbed material being recovered by changing the experimental conditions (see Fig. 4).

### 6.1 Coupling Procedure

CNBr-activated Sepharose-4B was obtained from Pharmacia and prepared according to the manufacturer's instructions. Dry gel was swollen and washed for 15 min on a sintered glass filter (Whatman, Maidstone, U.K.) with  $10^{-3}$  M HCl solution (200 ml/g of gel). The substance to be coupled (up to 10 mg protein/gm of gel) was first dialyzed against 0.1 M borate buffer pH 8.0 containing 1M NaCl and 0.02% NaN<sub>3</sub>, and then mixed with the gel. The mixture was rotated end-over-end for 2-4 hours at room temperature or overnight at 4°C. Unbound material was washed away with the coupling buffer and any remaining active groups were blocked by treatment with 1 M ethanolamine at pH 8.0 for 2 hours at room temperature. Three washing cycles were used to remove non-covalently bound proteins, each cycle consisting of a wash in 0.1 M acetate buffer pH 4.0 containing 1M NaCl followed by a wash in borate buffer at pH 8.0. The gel was stored at 4°C in the borate buffer containing a bacteriostatic agent (0.02% NaN<sub>3</sub>).

### 6.2 Immunoabsorption procedure and specific Ab recovery

The protein coupled gel was packed in a suitably sized syringe barrel. The column was washed with at least one column volume of borate buffer or PBS containing 0.02% NaN<sub>3</sub>. The solution to be

absorbed was applied to the column. After the column was loaded, its end was closed and the reactants allowed to remain in contact for at least one hour. The unbound protein was eluted with PBS. Fractions of 3 mls were collected until the optical density ( $E_{1\text{ cm}}^{280\text{ nm}}$ ) of the effluent reached zero. The specific bound material was recovered by running 0.2 M glycine-HCl pH 2.8 containing 0.5 M NaCl through the column. Fractions of 1.5 ml were collected in tubes containing 0.5 ml of 2 M tris-HCl buffer pH 7.8 in order to rapidly adjust the final pH of the eluates to physiological levels. Elution was performed at room temperature. The eluates were analysed for protein concentration and specific activity. The protein containing fractions obtained with each buffer were pooled, concentrated and kept frozen for further studies.

#### B.7 MANCINI RADIAL IMMUNODIFFUSION TECHNIQUE

Human IgG, IgA and IgM concentrations in fluids were estimated by the Mancini radial immunodiffusion technique (Mancini et al., 1965) using Hyland commercial plates (L.C. Partigen plates, Hyland Lorne, Thetford, U.K.). In this procedure, the diffusion of antigen into agar containing the specific antibody results in the formation of a circular zone of precipitation at equivalence. The diameter of this zone is directly related to the concentration of the diffusing antigen.

Before addition of samples the plate lids were lifted to allow moisture in wells to dry (10-15 min at R.T.). Using a Hamilton syringe 4  $\mu$ l of antigen was delivered to each well. Reference standards were included in each batch of assays. The plates were incubated for a minimum of 48 hours at R.T. The diameters (D) of precipitation rings were measured using a magnifying eye-piece with a graduated scale. A standard curve was obtained by plotting on two cycle semi-log paper the

diameters against the protein concentration of the standards; the test values were read off this curve. The results were expressed in mg/ml.

## B.8 HAEMAGGLUTINATION ASSAY

The most important part of this assay was the bulk preparation of tetanus toxoid-coated glutaraldehyde-fixed erythrocytes. This produced a stock of coated cells which were stored at 4°C and retained sensitivity throughout the 3½ year period of the study. The method used was based on the technique of Nelson et al. (1973).

### 8.1 Glutaraldehyde fixing of cells

One unit (450 mls) of human group "O" Rh-negative blood collected in anticoagulant (citrate phosphate dextrose) was obtained from the blood bank. The red cells were washed (x3) in physiological saline and the packed cell volume (PCV) established after spinning at 1000 g for 5 min. A 2% (v/v) suspension was made in 1% glutaraldehyde in PBS pH 7.2 and incubated at 4°C for 30 min with rinsing every 10 min. The cells were washed 3 times in saline followed by 3 times in distilled water to get rid of haemolysed and non-fixed cells. After establishing the PCV as before, 1 ml of tetanus toxoid at 300 µg/ml and 0.5 ml of 0.5% (w/v) chromic chloride was added to 1 ml packed cells. After mixing, this was incubated at 25°C for 6 min, washed in saline (x3) and stored as a 20% suspension in distilled water at 4°C until used.

### 8.2 Assay

An aliquot (25 µl) of the samples under test was serially diluted two-fold in 2% BSA saline (v/v) in microtitre "U" bottom plates (Flow Laboratories, Irvine, Ayrshire). Two preparations of human anti-tetanus IgG for clinical use, with known concentration of specific anti-tetanus

antibody, were used as standards. These were human anti-tetanus IgG (SNBTS Protein Fractionation Centre, Edinburgh, U.K.) and Humotet (Wellcome, London, U.K.). Negative controls consisted of human serum negative for anti-tetanus antibody activity (when available), or diluting fluid. Standards, controls and samples were allowed to react with 25  $\mu$ l 0.6% suspension of tetanus-coated cells (in 2% B.S.A.-saline) for two hours at room temperature. Negative samples were characterised by a dot of cells in the bottom of the well. Samples containing specific antibody caused the antigen-coated cells to agglutinate. The last dilution of antiserum at which agglutination was visible was taken as the antibody titre. This titre was converted into international units by the following formula:-

$$\text{I.U./ml} = \frac{\text{titres of test} \times \text{known units of standard}}{\text{titre of standard}}$$

#### B.9 HAEMAGGLUTINATION INHIBITION ASSAY (HIA)

Tetanus toxoid activity was measured by two stage HIA. The sample to be tested (25  $\mu$ l) was two-fold serially diluted in 2% BSA-saline (v/v), and mixed with an equal volume of standard anti-tetanus IgG at 0.125 I.U./ml of antibody activity. The plate was mixed by tapping along each edge, then incubated at 37°C for 30 min. An equal volume of 0.6% tetanus-coated cells (section B.8.1) was added to all wells. The plate was mixed and reincubated at 37°C for a further hour. In the presence of tetanus toxoid the agglutination of the sensitized cells was inhibited. This was characterised by a dot of cells in the bottom of the well. The last dilution of tetanus toxoid at which the dot of cells was visible was taken as the titre.



## B.10 SEPARATION AND STORAGE OF SERA AND PLASMA

### 10.1 Mouse antisera

CBA mice were injected i.p. with 10  $\mu$ g of Wellcome tetanus toxoid. BALB-c mice were injected i.p. with either 10  $\mu$ g Wellcome or Connaught tetanus toxoid. Identical booster injections were subsequently given on days 28 and 56. Six mice from each treatment group were bled on day 0 and thereafter every fortnight until day 70. The serum of each individual mouse was separated after centrifugation, and stored frozen at -20°C.

### 10.2 Human plasma

Tetanus toxoid-boostered donors were plasmaphoresed. A sample of each plasma was assayed for anti-tetanus antibody activity by ELISA (C.1) and haemagglutination (HA). The high titre plasma were clotted with commercial bovine thrombin (Parke Davis, U.S.A.; final concentration of 10 units/ml) by mixing and allowing to stand at 37°C for 2 hr. The clots were squeezed and the expelled sera were left stirring at 4°C overnight during which time further clotting was occasionally observed. The following day the anti-tetanus sera were divided into 25 ml aliquots in glass McCartney bottles, and centrifuged at 200 g for 15 min. The clear supernatant was recovered and stored at -20°C in 0.1% NaN<sub>3</sub>.

B.11 Source and properties of antibodies used

Antibody	Produced in	Form	Source
Anti-human IgM " " IgA " " IgG	Sheep	In agar in radial immunodiffusion (RID) plates	L.C. "Partigen" RID-plates Hyland Co., Thetford, U.K.
Anti-mouse IgM " " IgA " " IgG1 " " IgG2a " " IgG2b " " IgG3	Rabbit	Monospecific antisera to mouse Ig - isotypes prepared by solid phase immunoadsorption	Litton Bionetics Inc., Kensington, Maryland, U.S.A.
Anti-mouse Ig	Rabbit	Isolated by salt precipitation and ion - exchange chromatography	DAKO, West Byfleet, U.K.
Alkaline Phosphatase-conjugated anti- Ig	Sheep	Affinity purified anti-mouse Ig (H+L)	New England Nuclear Boston, Massachusetts.
	Goat	Affinity purified anti-rabbit Ig (H+L)	TAGO, Inc., Immunodiagnostic Reagents, Burlingome, Calif.
	Goat	Affinity purified anti-human Ig (H+L)	Miles Laboratories Ltd., Slough, U.K.
Tetanus antibody standard serum	Human	Whole serum	West of Scotland Blood Transfusion Service, standardised by NIBSAC, London, U.K.
Human anti-tetanus Ig	Human	Cohn Fraction II	Protein Fractionation Centre, Edinburgh, U.K.
Human anti-tetanus Ig	Human	Cohn Fraction II	Wellcome Research Laboratories, Beckenham, U.K.

## B.12 MATERIALS

Common laboratory reagents were obtained from BDH Chemicals Ltd. (Poole, U.K.). This is a list of the specific materials where their sources have not been mentioned in the text.

Tetanus vaccine (Well<sub>1</sub>-TT): Wellcome Foundation Ltd., Crewe, U.K.

Tetanus toxoid (Well<sub>2</sub>-TT): Wellcome Research Laboratories, Beckenham, Kent, U.K.

Tetanus toxoid (Conn-TT): Connaught Laboratories, Willowdale, Ontario, Canada.

p-Nitrophenyl disodium phosphatase: Sigma Chemical Co. Ltd., Poole, Dorset, U.K.

Radiochemicals (<sup>125</sup>I and <sup>131</sup>I): Amersham International Plc, Amersham, Bucks, U.K.

Ammonium Sulphate: May and Baker Ltd., Manchester, U.K.

RPMI 1640 - tissue culture medium: GIBCO Europe Ltd., Paisley, U.K.

Penicillin/Streptomycin: GIBCO Europe Ltd., Paisley, U.K.

Hypoxanthine: GIBCO Europe Ltd., Paisley, U.K.

Thymidine: GIBCO Europe Ltd., Paisley, U.K.

Aminopterin: Sigma Chemical Co. Ltd., Poole, Dorset, U.K.

Lignocaine: Sigma Chemical Co., Ltd., Poole, Dorset, U.K.

Foetal Calf Serum (FCS): Flow Laboratories Ltd., Irvine, U.K.

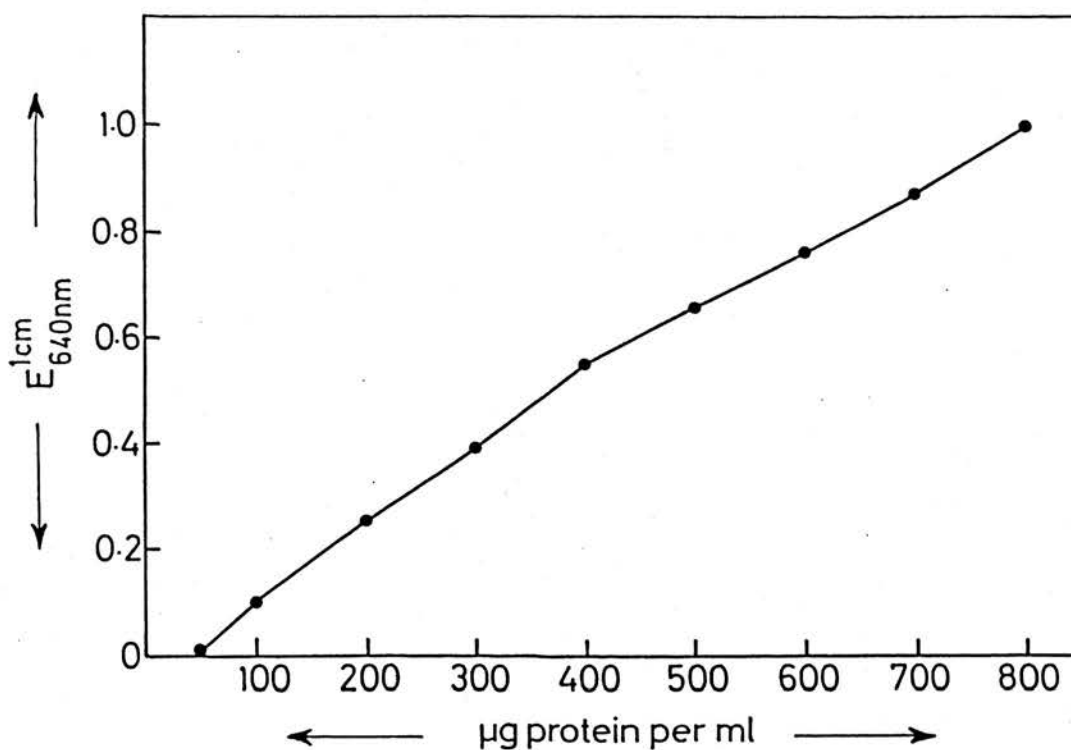
Fungizone: Squibb & Sons Inc., Princeton, U.S.A.

Karomycin: Winthrop Laboratories, Surbiton-upon-Thames, U.K.

Leukopak (Nylon Wool): Fenwall Laboratories, Madison, Wisconsin, U.S.A.

Tissue Culture flasks and Flat-bottomed plates: Corning Ltd., New York, U.S.A.

FIG. B-1: Standard calibration curve for determination of total protein by Folin-phenol method



A standard curve is constructed by testing a range of protein concentrations of BSA. This is then used to estimate the total protein values of unknown samples.

TABLE B-1

STANDARDISATION OF PROTEIN BY FOLIN-PHENOL METHOD

Standard B.S.A.	O.D.1	O.D.2	$\mu\text{g/ml}$
1	0	0	25
2	0.01	0.01	50
3	0.09	0.1	100
4	0.26	0.27	200
5	0.38	0.38	300
6	0.55	0.55	400
7	0.65	0.65	500
8	0.76	0.76	600
9	0.84	0.86	700
10	1.0	1.0	800

Various dilutions of the protein standards tested in duplicate show great similarity of the O.D. values. Using these values a standard curve was constructed (see Fig.1) and used to estimate the total protein values of unknown samples.

TABLE B-2

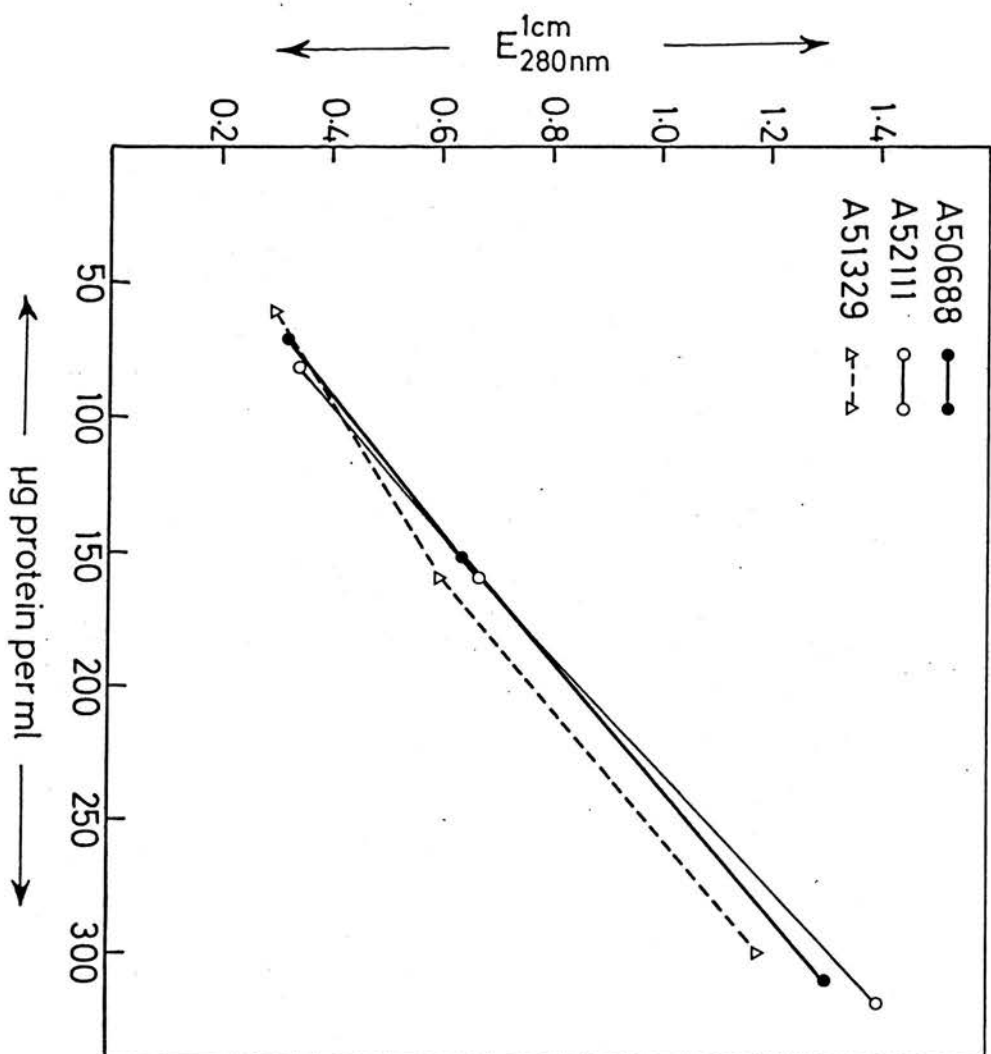
A COMPARISON OF THE FOLIN-PHENOL PROCEDURE AND DIRECT UV  
ABSORBENCE AT 280 n.m. FOR THE MEASUREMENT OF THE PROTEIN CONTENT  
OF BATCHES OF TETANUS TOXOID

Tetanus-Toxoid	Dilution	O.D. (Folin)	(a) $\mu\text{g/ml}$	1cm E 280n.m.	(b) $\mu\text{g/ml}$
Batch: (A50688)	Neat	0.41	310	1.3	310
	1:2	0.18	152	0.64	152
	1:4	0.045	72	0.32	76
	1:8	0	—	0.17	41
	1:16	0	—	0.09	21
Batch: (A52111)	Neat	0.42	316	1.4	333
	1:2	0.19	158	0.67	160
	1:4	0.065	82	0.34	81
	1:8	0	—	0.18	43
	1:16	0	—	0.1	24
Batch: (A51329)	Neat	0.38	300	1.2	286
	1:2	0.17	150	0.59	140
	1:4	0.03	62	0.31	74
	1:8	0	—	0.17	41
	1:16	0	—	0.09	21
Batch: (A52109)	Neat	Not done		1.3	310
	1:2	"	"	0.64	152
	1:4	"	"	0.32	76
	1:8	"	"	0.16	38
	1:16	"	"	0.08	19

(a) The protein concentrations were measured by using the standard curve (Fig.1) of Folin-phenol procedure.

(b) The values were obtained by direct U.V. measurement of protein solutions using an extinction coefficient for tetanus toxoid of 42. The values obtained were very similar using both techniques (a) and (b).

FIG. B-2: Total protein concentration of various batches of tetanus toxoids by Folin-phenol and U.V. absorbance at 280 n.m.



The protein concentrations of various batches of Wellcome tetanus vaccine were similar.

TABLE B-3

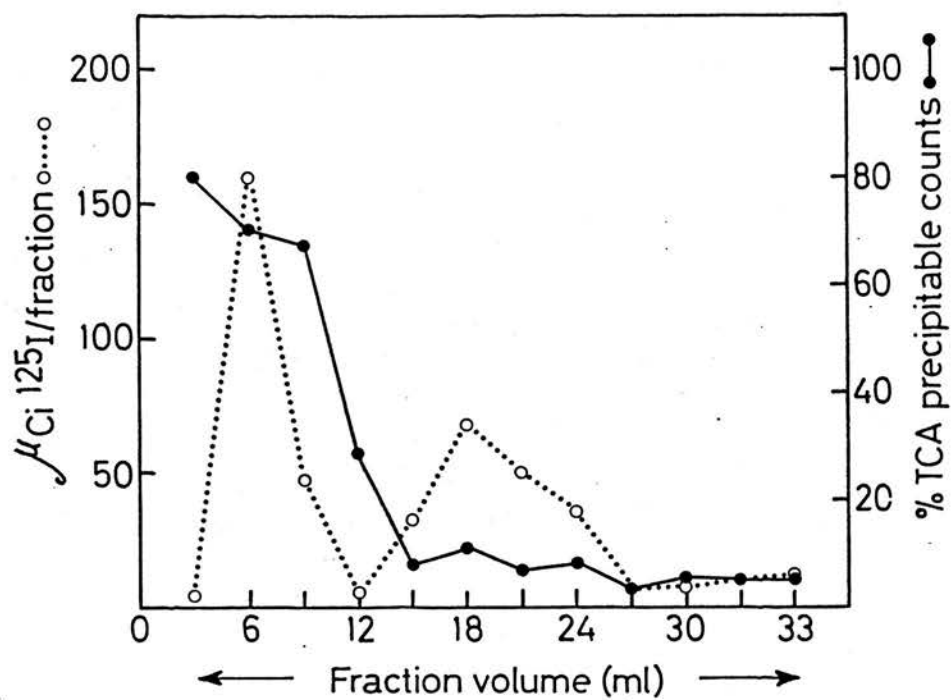
EFFICIENCY OF RADIO-IODINE LABELLING PROCEDURE

Sample	% TCA precipitability
Tetanus toxoid (Wellcome 1)	77%, 70%, 63%, 68%, 78%, 61%, 72%
Tetanus toxoid (Wellcome 2)	88%
Tetanux toxoid (Connaught)	90%, 88%
IgG	92%
B.S.A.	93%

The values represent results of separate radio-iodine labellings.  
The labelling efficiency varies depending on the protein being  
labelled.

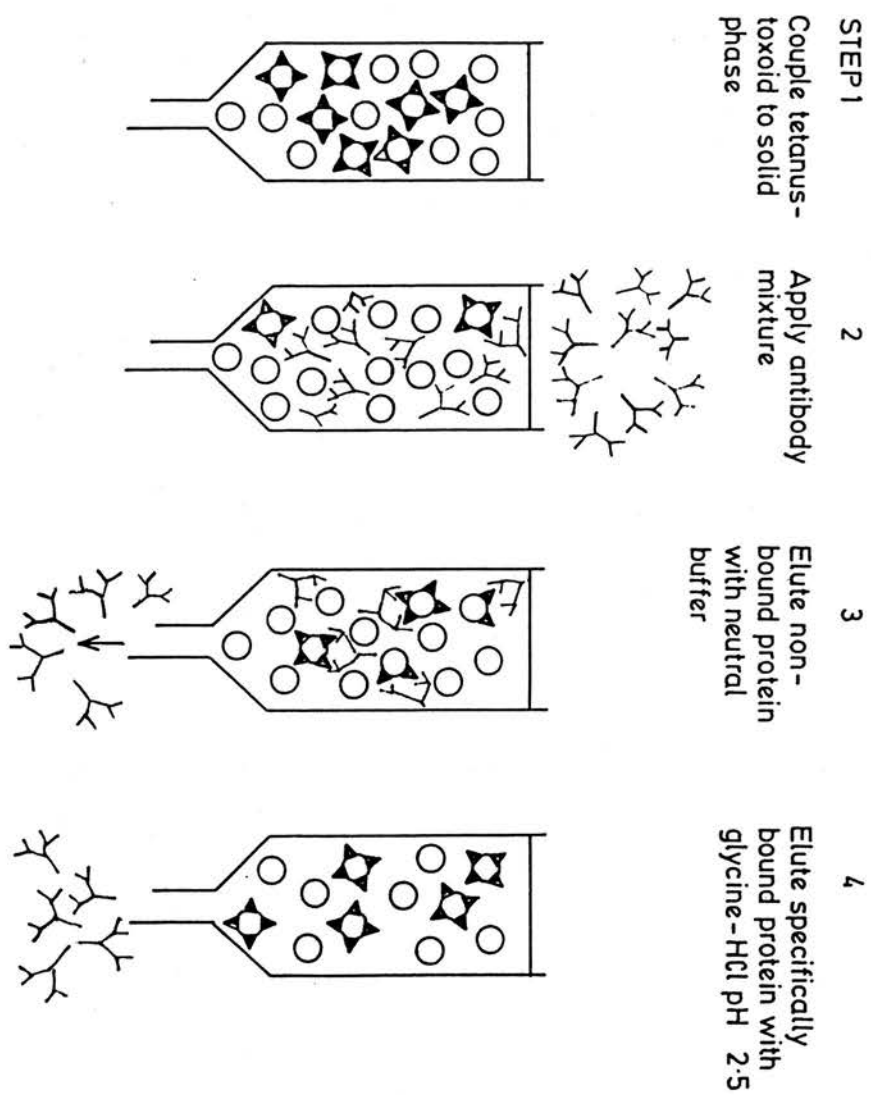


FIG. B-3: Typical elution profile of radioiodinated tetanus toxoid on G-25 Sephadex



In this separation fraction 2 was chosen for further study

**FIG. B-4: Principle of immunoadfinity purification of antitetanus antibodies**



C.1 CHAPTER 1

DEVELOPMENT OF ASSAYS FOR MOUSE AND HUMAN

ANTI-TETANUS ANTIBODIES (ELISA)

## C.1 DEVELOPMENT OF ASSAYS FOR MOUSE AND HUMAN ANTI-TETANUS ANTIBODIES AND FOR ISOTYPE ANALYSIS

### Introduction

Antibodies to tetanus-toxoid are a reliable indicator of host protection, and the measurement of anti-tetanus antibodies is of value in assessing the immune status of individuals at risk, for the selection of potential donors, for the production of anti-tetanus immunoglobulin, and in monitoring the efficiency of vaccination programs. The advent of hybridoma technology has also necessitated the development of sensitive assay procedures which can be applied to the rapid screening of larger numbers of samples of limited volume or with low concentration of antibody.

An Enzyme Linked Immunosorbent Assay (ELISA) had to be developed to meet all the above mentioned purposes. It is a simple and sensitive method for the quantitative determination of antibody to tetanus toxoid permitting the detection of anti-tetanus antibody levels as low as  $5 \times 10^{-4}$  I.U./ml.

Prior to its development several techniques were available for measuring anti-tetanus antibodies. The first and most widely used method for detection of immunity to clinical tetanus was (and still is) the toxin neutralization test, developed by von Behring and Kitasto (1890). This test measures the ability of tetanus toxin antibodies to protect mice from a standard inoculum of tetanus toxin. It is a time consuming test, and it takes five days to analyse a serum sample. In addition only antibodies of the IgG class seem to be capable of neutralizing tetanus toxin in mice (Ourth et al., 1977; Winsnes et al., 1979). Nevertheless, it still remains a suitable in vivo test, but it cannot satisfy routine screening need. Other techniques used

include counter-immunoelectrophoresis (Barr et al., 1975; Winsnes, 1979), an automated latex particle technique (De saint et al., 1975) and a direct haemagglutination procedure (Nelson et al., 1973). Results from most of these techniques are expressed in the form  $>$  or  $\leq$  6.0 I.U./ml (De saint et al., 1975).

More sensitive assays exist. These include the reversed RIA technique which can detect levels greater or equal to 0.01 I.U./ml (Bernath et al., 1974 and Dow et al., 1983). However like most RIA assays they require long incubation periods. For example, the RIA procedure using protein-A (Haberman et al., 1973) takes 4 days for an assay and uses a large number of tubes rather than one microtitre plate (96 wells). Another disadvantage of RIA as compared to ELISA is that they generally require more purified reagents than ELISA technique (Wang Ai Sha et al., 1982). In addition, there are the problems associated with the handling of hazardous radioactive materials, and requirement of expensive and special equipment such as gamma counter.

The ELISA to be described is a simple, safe, cheap, rapid and sensitive assay. It takes 4 hours to perform and can detect anti-tetanus antibody as low as  $5 \times 10^{-4}$  I.U./ml.

## 1.2 Development of ELISA for quantitation of total anti-tetanus antibody (Human)

### 1.2.1 Introduction

The technique used was based on the ELISA procedure first described by Enzvall et al. (1972). The steps involved in this assay are summarised in Fig.1a. In the initial step the wells of a microtitre plate are coated with a dilute solution of antigen (in this

case tetanus toxoid). The antibody preparation under test is then added to the plate. This is followed, after incubation and washing by an enzyme conjugated (in this study alkaline phosphatase) antibody directed against the appropriate immunoglobulin. Finally, after a further period of incubation and subsequent washing the substrate (p-Nitrophenyl disodium phosphatase) is added. This reacts with conjugated antibody liberating paranitrophenyl which exhibits a yellow colour. The intensity of colour is then measured spectrophotometrically in a Multiscan at 405 nm (Dynatech, Massachusetts, U.S.A.), thus indicating the amount of specific antibody in the sample.

This ELISA procedure was used routinely to assay antitetanus antibody levels in serum samples of immunized donors, tetanus infected patients, crude tetanus IgG preparations, purified tetanus antibody isolated by means of affinity chromatography, and in screening human monoclonal antibodies to tetanus toxoid.

#### 1.2.2 Material and Method

##### 1.2.2.1 The development of a satisfactory ELISA procedure

Different procedures for coating the antigen (tetanus toxoid) to the plates, and for performing the assays were investigated in order to establish the optimum procedure. This was subsequently modified for semi-automated routine use. The concentrations of tetanus toxoid used to coat microelisa plates were 500, 100  $\mu\text{g/ml}$ , and thereafter two-fold dilutions to 6.25  $\mu\text{g/ml}$ . Each concentration of tetanus toxoid was assayed against different concentrations of tetanus-IgG standard (range 0.5-0.0005 I.U./ml doubling dilution). These concentrations were the

same in the 5 different procedures tried. The variables tested in the procedures are listed in Table 1.

#### 1.2.2.2 Choice of microtitre plate for ELISA

Microtitre plates from four different manufacturers were used, these namely Costar, Dynatech, Optiken and Nunc (for details, see Table 2). The ELISA assay performed was procedure No.4 (Table 1).

#### 1.2.2.3 Determination of optimal concentration of antigen

ELISA procedure (No.4) was carried out on plates coated with the various dilutions of tetanus toxoid (range of 565-7.0  $\mu\text{g/ml}$ ). The results ( $A^{405}$ ) obtained from titration of tetanus-IgG standard tested on each dilution of tetanus toxoid were compared.

#### 1.2.2.4 Investigation of the effect of excess washing on the test results

The washing of the plates between the various steps of ELISA was done using an automatic plate washer (Dynatech). One group of plates were washed 4 times post conjugate and 2 times for other washes. In the other plates the number of washes were doubled and results were compared.

#### 1.2.2.5 Investigation of the effect of the incubation period with substrate

After addition of substrate (1.0 mg/ml) to a duplicate set of plates, the results ( $A^{405}$ ) were monitored after incubating (37°C), one set of plates for 30 min and the other for 60 min.

#### 1.2.2.6 Determination of the optimum concentration of conjugate

The anti-tetanus standards were assayed by using 1:500, 1:800 and 1:1000 dilutions of the conjugate in PBS containing 0.05% Tween 20.

#### 1.2.2.7 Investigation of the effect of storage and coating procedure on results

A series of plates which were coated and stored under different conditions were tested by assaying the three different anti-tetanus standards, and serum samples from tetanus boosted donors. These were as follows:-

Plate (a): coated with tetanus toxoid (50 µg/ml) and stored at 4°C with the fluid in the wells. The plates were tested every week for 5 weeks.

Plate (b): coated and incubated overnight with tetanus toxoid solution in the wells, and used the next day.

Plate (c): coated with tetanus toxoid and incubated first at 37°C for 3 hrs, and then at 4°C overnight. Next day the plates were washed and dried. One of these plates was compared to the above two plates and the rest were sealed and stored at 4°C. These plates were used over a period of 10 months.

#### 1.2.2.8 Selection of the best anti-tetanus standard

For the purpose of applying the ELISA technique for routine testing of sera of tetanus immunized donors, the assay had to be standardized. This was done by comparing the 3 anti-tetanus standards with known level of anti-tetanus antibody activity (I.U./ml). The three standards were two-fold serially diluted and assayed in plates



coated in the three different ways (a,b, & c). The plasma samples were tested at single dilution (1:10,000), and the results were read against each of the anti-tetanus standard curves. The anti-tetanus antibody level was expressed as I.U./ml.

#### 1.2.2.9 Determination of non-specific binding in ELISA by using normal human serum (NHS)

Human serum negative in anti-tetanus antibody was included in each assay as a negative control. It was also used as a blank in the multiscan microtitreplate reader.

To ensure there was no non-specific binding at higher NHS concentration, tests were carried out using 10% as well as 1% NHS. A series of two-fold dilutions and a few different concentrations of anti-tetanus standard (NIBSAC) were read against both NHS concentrations.

#### 1.2.1.10 Heating of the plasma samples prior to ELISA

A series of plasma samples were preheated at 56°C for 30 min and assayed in parallel with unheated samples. The samples were two-fold serially diluted starting with 1:10 dilution.

### 1.2.3 Results and Discussion

#### 1.2.3.1 The development of a satisfactory ELISA procedure

Five different procedures were tried in order to select a simple, rapid and sensitive assay for detection of anti-tetanus antibody. Four out of the 5 procedures tried gave satisfactory results, as illustrated in Fig.1b.

For further studies and as a routine assay, the procedure 3 was chosen since it was the simplest, fastest and exhibited good sensitivity. Although the Ag-coating and assay can be completed in the same day, this procedure was further simplified for routine use by coating hundreds of microtitre plates in advance. This involved an initial incubation at 37°C for 3 hrs with tetanus toxoid followed by further incubation at 4°C overnight. The next day the plates were shaken dry, sealed and stored in batches of three at 4°C. Plates prepared and stored in this manner had been found to be satisfactory for at least one year after initial preparation, anti-tetanus antibody titres as low as  $5 \times 10^{-4}$  I.U./ml still being detectable (see Fig.2). The assay procedure using the stored tetanus toxoid coated plates takes only 4 hours, whereas the most recent radioimmunoassay techniques for tetanus antibody measurement takes 3-4 days to perform (Dow et al., 1983).

#### 1.2.3.2 Choice of microtitre plate for ELISA

Microtitre plates can be purchased from a number of different manufacturers. Unfortunately, however, the results of ELISA assays appear to be dependent on the source of manufacture. To find the plate which would give the most sensitive and reproducible results, ELISA assays were performed on plates from a number of different manufacturers (Fig.3 and Table 2). The best results were obtained on plates made by "Costar" and "Dynatech", however as the "Dynatech" plates were cheaper, they were chosen for further assays. Plates manufactured by "Optiken" exhibited high anti-tetanus antibody titre, but results were irreproducible. The reverse was true on plates made by "Nunc" (Table 2).

#### 1.2.3.3 Determination of the optimal concentration of antigen

The assay appeared suitable over a wide range of antigen concentrations, results of which are shown in Table 3 and Fig.4. A concentration of 50 µg/ml of tetanus toxoid was selected for routine use. Since a wide range of antigen concentrations can be used to coat the plates with test sensitivity still maintained, it is not necessary to detect the optimal concentration each time, as long as the same tetanus toxoid is used. It is advisable that the optimal concentration be checked if tetanus toxoid from other suppliers or any other antigens are used.

#### 1.2.3.4 Effect of excess washing on the test results

As observed in Table 4 and Fig.5, excess washing of the plates does affect the test results. The absorbance at 405 n.m. ( $A^{405}$ ) of anti-tetanus standard decreases slightly as the number of washes increases, indicating that some protein can be detached from the wells by excess washing.

#### 1.2.3.5 Effect of time of incubation with substrate

By increasing the incubation time of the substrate at 37°C, the intensity of the colour produced increased. As the results in Table 5 and Fig.6 indicate, there was a two-fold increase in optical density of the anti-tetanus standard when the incubation period with the substrate was doubled. This confirms that one hour incubation with substrate had been a good choice, as it gives good sensitivity. But the shorter incubation period should not have a dramatic effect on the overall results if a standard is included in each assay. The calibration curve thus obtained is used to evaluate the test results.

#### 1.2.3.6 Effect of different concentrations of conjugate on test results

The optimal concentration of conjugate was determined using different dilutions of the conjugate. The results in Table 6 and Fig.7 (a,b,c & d) confirm that the 1:500 dilution of conjugate is the best concentration giving the most sensitive and most reproducible results, the  $A^{405}$  increasing linearly with the concentration of anti-tetanus antibody standards.

#### 1.2.3.7 Effect of the storage and coating procedure on results

In order to obtain an accurate and reproducible assay which can be used on a routine basis, it was essential to find a reliable coating and storage procedure for the plates. In this trial anti-tetanus standards and serum samples were tested using plates which were coated and stored under different conditions (see page 68). The sensitivity of the test seemed to increase (Table 7, Fig.8 and Fig.9(a)), each week using plates which were stored with tetanus toxoid solution in the well (plate (a)). At the higher antibody concentrations, the results increased greatly with storage to exceed the range which the multiscan can measure ( $A^{405} = > 2.0$ ); this is shown in Table 7 (see results of the 5th week). This could have been due to evaporation and thus further concentration of tetanus toxoid solution in the wells and/or further absorption of the antigen by the solid phase.

The results using plate (b), which was coated with the tetanus toxoid solution in the wells but used immediately the next day were better than in plate (a). Using these plates the antibody levels being readable by multiscan up to 0.5 I.U./ml (Table 9) as compared to 0.01 in plate (a). However, routine assays using this coating procedure may

not be practical for urgent samples because the plates must be coated one day before the assay, and can not be stored for later use, since the same problem observed in plate (a) would arise.

Coating and storage as in plate (c) proved to be the best (that was coating and incubating with tetanus toxoid solution in the wells first at 37°C for 3 hrs and then overnight at 4°C. Next day after washing and drying the plates, they were sealed properly and stored until use). As it is shown in Fig.9(c), Tables 8 and 9, the results of anti-tetanus standards in this plate were much better to that observed above. The three standard curves were parallel and gave a wider linear range ( $A^{405} = 0.08-1.2$ ) than they did in plates (a) and (b).

A quantitative measurement of antibody levels in all of the 8 serum samples from tetanus-boosted donors was achieved by using only one single serum dilution of 1:10,000 (Table 8). The antibody level (I.U./ml) of the same sample read against three different standard curves were the same, except when using plate (b).

In Fig.9(d) the standard (NIBSAC) curves from differently coated plates are redrawn. This shows that plate (a) results are above the acceptable/readable range of multiscan, and plates (b) and (c) results are acceptable and reproducible, but since in assays using plate (b) two days are needed, plate (c) proves to be the most suitable and practical procedure for coating and especially storage, since they have a long shelf life.

#### 1.2.3.8 Selection of the best anti-tetanus standard

From Fig.9 (a,b and c) and Table 9, it is apparent that the "NIBSAC" standard is the most suitable for the assay, followed by "Humotet". A more linear standard curve was obtained with "NIBSAC",

and its linear working range was wider than the other two standards when assayed in three differently coated plates (Table 9). In addition, as the "NIBSAC" standard is a pool of anti-tetanus plasma, it would be best in routine testing of antiserum of tetanus immunized donors. The "PFC" standard used in these tests appears to give irreproducible results. This may be due to batch to batch variability of the tetanus IgG concentration, this being in the range of 40-180 I.U./ml (according to PFC). All the batches are labelled 250 I.U./2.5 ml which makes this standard a rather unreliable one.

In Fig.10, the three standards have been two-fold serially diluted. The "NIBSAC" standard curve approaches linearity between doubling dilutions of 7 to 11. This demonstrates that fewer dilutions of this standard can be used and maintain the same linear range, giving the possibility of quantifying specific antibody concentration at a single reading within that region (as in Table 8). However, it is advisable to use other lower dilutions as well in order to detect lower concentration of anti-tetanus antibody in sera of non-immunized donors. In this connection, it is interesting to note that antibody levels as low as  $5 \times 10^{-4}$  I.U./ml can be detected using the ELISA assay which has been developed (Fig. 9).

#### 1.2.3.9 Determination of non-specific binding in ELISA using normal human serum (NHS)

As a routine it is important to include in each assay a serum negative in anti-tetanus antibody (NHS), as a negative control to look for any non-specific reaction. NHS should give absolutely no colour reaction when it is assayed under the same conditions as positive sera. The multiscan ELISA-plate reader should be blanked using 1% NHS (rather than BSA or substrate alone as some do), and then the test sample and

standards read against it. In the test which was carried out using higher NHS concentrations (10%) as well as 1%, there were no differences between the two test results (Table 10 and Fig.11). Both concentrations of NHS were negative by the ELISA, indicating that there is no non-specific binding using this technique.

#### 1.2.3.10 Effect of heating plasma sample prior to ELISA

An interesting finding has been reported by Vos et al. (1978), who used an ELISA technique to quantify antibody to LPS. They found that at low serum dilutions(i.e. 1:10), the antibody level was lower than at higher dilution (i.e. 1:100 or, antibody titre higher in more diluted sample). This inhibition at higher concentrations was thought to be caused by a heat-labile serum component, as heating of the serum at 56°C for 30 min prevented this phenomenon. Although I did not encounter this problem to the same extent, a more linear dilution curve was obtained by pre-heating the anti-tetanus plasma (Fig.12). The results also showed an increase in reaction at higher concentrations of heated sample.

#### 1.2.4 Conclusions

1.2.4.1 The enzyme-linked immunosorbent assay developed to measure anti-tetanus antibodies is rapid, sensitive and capable of measuring anti-tetanus antibody level as low as  $5 \times 10^{-4}$  units/ml.

1.2.4.2 The use of microtitre plates instead of tubes is economical.

- 1.2.4.3 A wide range of protein concentration can be coated to the solid phase. Antigen-coated plates can be stored for routine use for a long period of time (10 months so far).
- 1.2.4.4 There was no non-specific binding observed in this technique.
- 1.2.4.5 A wide range of standards can be used, but NIBSAC has proven to be the most suitable standard.
- 1.2.4.6 It is ideal for screening large numbers of samples. The specific antibody can be quantified at a single serum dilution (1/10,000).
- 1.2.4.7 It is safe as compared to radioimmunoassay.
- 1.2.4.8 It can be used to measure all immunoglobulin classes and subclasses.
- 1.2.4.9 It can be applied to measure any other antibody quantitatively, as long as the relevant antigen can bind readily to the polyvinyl solid phase.

### 1.3 Development of ELISA for quantitation of mouse anti-tetanus antibody

#### 1.3.1 Introduction

This assay was developed to quantitate anti-tetanus total antibody level in sera of immunized mice. This was done to measure antibody



responses of mice to various doses of tetanus toxoid, and prior to fusion for production of mouse monoclonal antibodies to tetanus toxoid. The assay was used extensively in screening large numbers of hybridoma supernatants, and subsequently in measuring monoclonal antibody level in mouse ascitic fluid.

The assay was slightly modified from the ELISA previously described (Section C.1).

### 1.3.2 Materials and Methods

Materials and methods were similar to those used in the ELISA for human anti-tetanus antibody (Section C.1), except for the conjugate. The conjugate used in this assay was sheep alkaline phosphatase conjugated antibody directed against mouse Ig (NEN, Southampton, U.K.). In order to determine the optimal concentration different dilutions of this conjugate were tested.

The antibody containing samples under test were examined at a range of doubling dilutions, the initial dilution depending on the sample being examined. The results after correction for background (1% NMS) were expressed as  $\log_2$  titres of antibody. The end point in this assay was the dilution giving a reading at  $O.D_{405}$  of  $\geq 0.1$ .

### 1.3.3 Results and Discussion

The ELISA assay for measurement of the total tetanus antibody of mice to tetanus toxoid was identical to that developed to measure the total tetanus antibody in human (Section C.1). The only difference was that the conjugate chosen for this assay was directed against total mouse Ig. Most commercially available AP-conjugated anti-Ig are not just heavy chain specific but contain some light-chain activity as well. Therefore assays performed with such reagents measure IgM and

IgA antibodies as well as IgG.

The optimal concentration of this conjugate was determined using pools of serum from both normal and immunized mice. The results are shown in Table 11. A conjugate dilution of 1:500 was selected for routine assay, since it showed optimum specificity and sensitivity.

FIG. C.1-1a

ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA) FOR DETECTION OF ANTITETANUS ANTIBODY

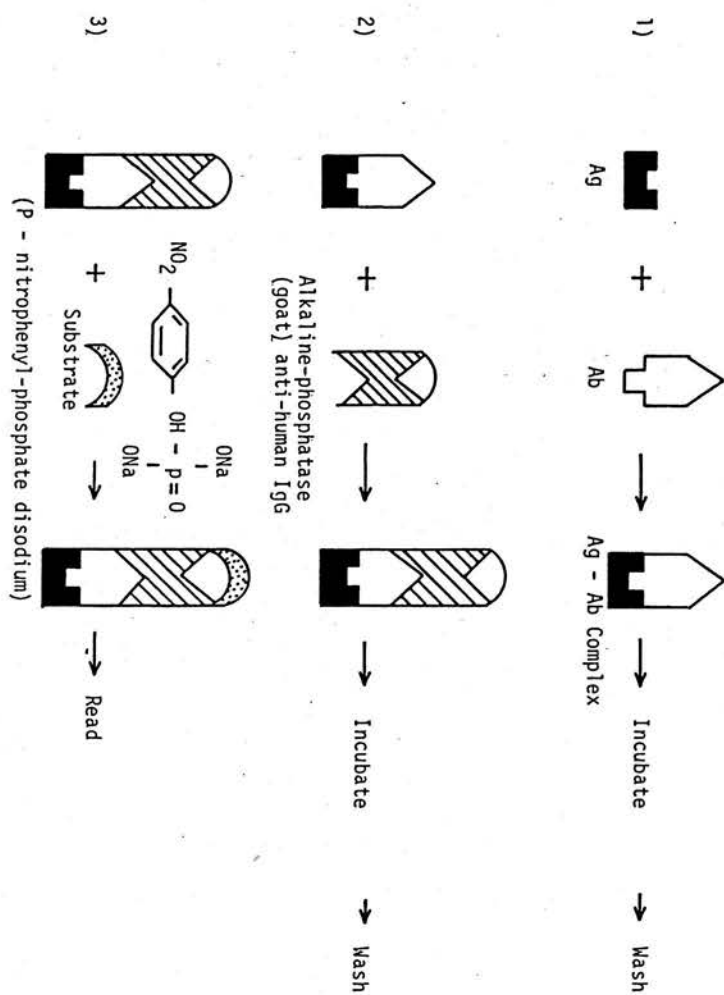


TABLE C.1-1

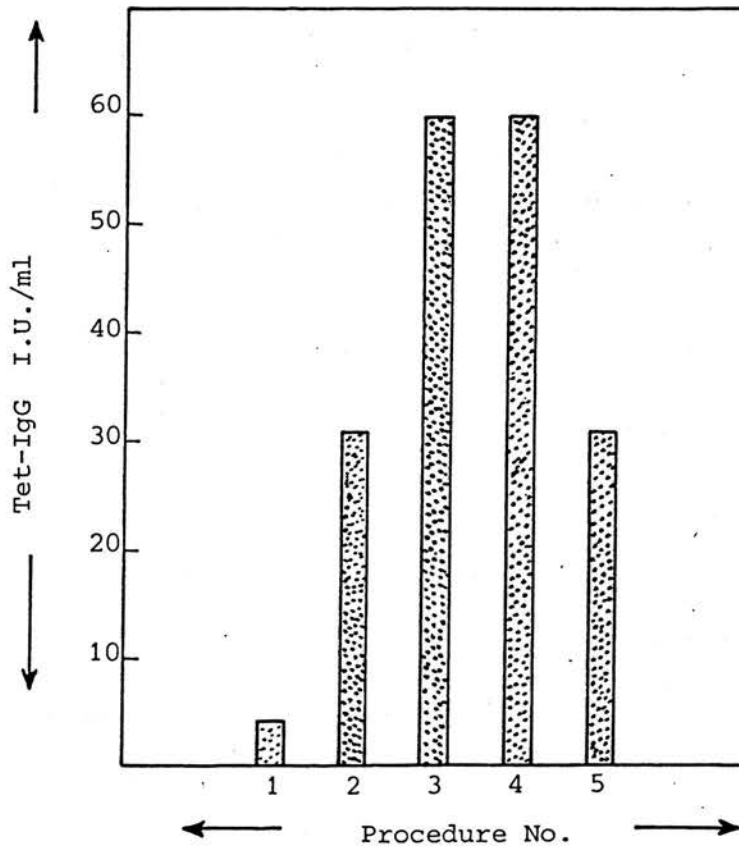
Variables tested for development of the ELISA technique

Variable	Procedure 1	Procedure 2	Procedure 3	Procedure 4	Procedure 5
<u>Ag Coating:</u>					
Ag-diluent	Distilled water	sodium-bicarbonate (0.05M, pH 9.6)	1% B.S.A./P.B.S.(v/v) (0.15M, pH 7.2)	1% B.S.A./P.B.S. (v/v)	Distilled Water
Ag - incubation time and temperature	3 hrs., 37°C O/N, 4°C	3 hrs., 37°C O/N, 4°C	1 hr., 37°C	3 hrs., 37°C O/N, 4°C	O/N, R.T.
Glutaraldehyde fixed	-	-	-	-	+ 30 min., at R.T.
Preincubated with B.S.A. (1% in P.B.S. v/v)	-	-	-	-	+ Dried O/N at R.T.
<u>Assay:</u>					
Washing buffer	Distilled Water	Saline (0.9% NaCl w/v)	Saline	0.05% tween - P.B.S. (v/v)	Distilled Water
Sample diluent	0.05% tween - P.B.S.	0.05% tween - P.B.S.	1% B.S.A.- P.B.S.	1% B.S.A.- P.B.S.	0.5% B.S.A. + 0.05% tween - P.B.S.
Sample incubation time and temperature	3 hrs., 37°C	4 hrs., R.T.	1 hr., 37°C	4 hrs., R.T.	1 hr., 37°C
Preincubation with 1% B.S.A./P.B.S.(v/v)	+ 1 hr., 37°C	-	+ 1 hr., 37°C	-	-

FIG. C.1-1b

A COMPARISON OF THE SENSITIVITY OF VARIOUS

ELISA PROCEDURES



Results of the different ELISA procedures (see Table C.1-1) used to establish the most sensitive method for measuring anti-tetanus antibody levels (I.U./ml). Procedures 3 and 4 showed the highest sensitivity.

FIG. C.1-2

STANDARD CURVE FOR ANTI-TETANUS DETERMINATION

(plates were coated 10 months prior to the assay)  
standard = NIBSAC containing 30 I.U./ml (anti-tetanus serum)

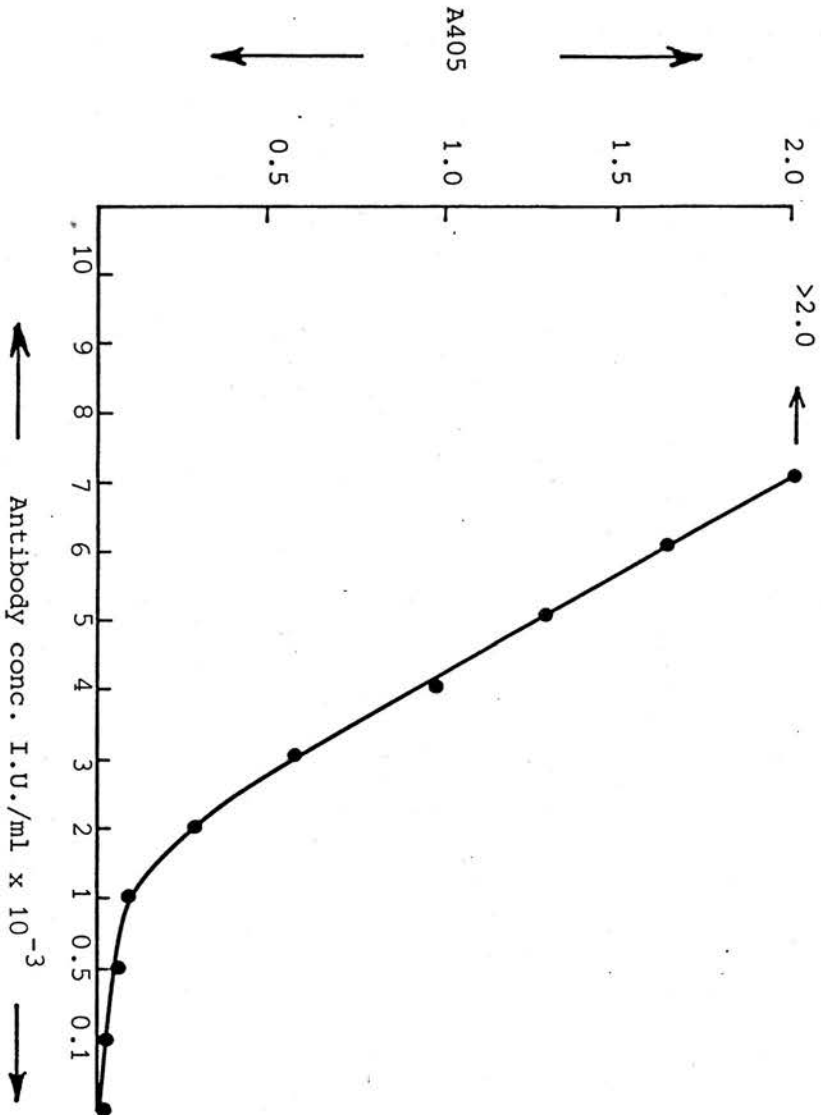


TABLE C.1-2

ELISA - titre of antitetanus - Ab measured in tetanus - toxoid immunized mouse serum using microtitre plates from different manufacturers

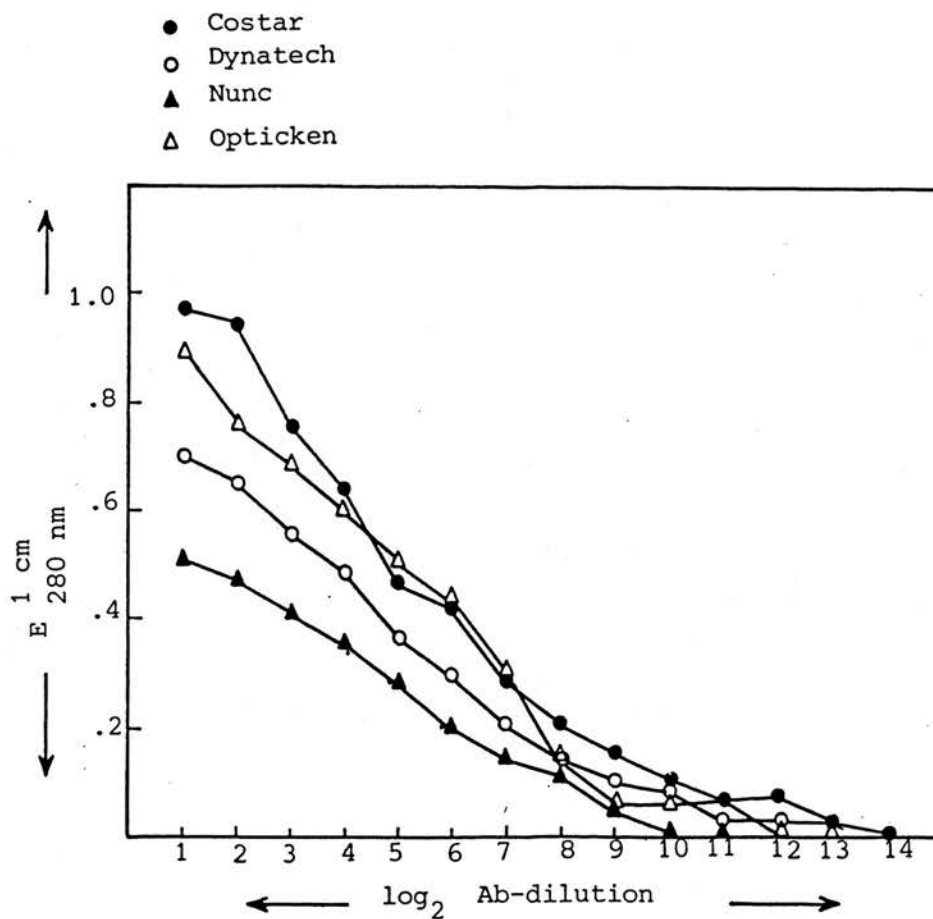
ELISA Microtitre plates	Ab- titre		
	(1)	(2)	(3)
NUNC Immuno Plate I for ELISA	1 : 12,800	1 : 12,800	1 : 12,800
COSTAR - Serocluster EIA plate, Half-Area (A/2)	1 : 51,200	1 : 51,200	1 : 51,200
OPTIKEN DISH (nbl)	1 : 12,800	1 : 102,400	1 : 25,600
DYNATECH Microeliza	1 : 25,600	1 : 25,600	1 : 51,200

1. "Nunc" plate (Beveridge, U.K.) shows reproducibility of result, but the Ab- titres were lower than observed with other plates.
2. "Costar" plate (Massachusetts, U.S.A.) demonstrates reproducibility and sensitivity and is therefore the best plate of the four plates to be used in this ELISA technique.
3. "Optiken" plate (Northumberland, U.K.) was highly irreproducible.
4. "Dynatech" plate (Sussex, U.K.) is relatively reproducible, and the Ab- titres are higher than other plates with the exception of "Costar" plate.

FIG. C.1-3

COMPARISON OF 4 DIFFERENT MICROTITRE PLATES IN ELISA

(coated 5 months previously)



Although the best results were obtained using "Costar" plate, "Dynatech" was chosen for routine use, since these plates were cheaper and gave more reproducible results.



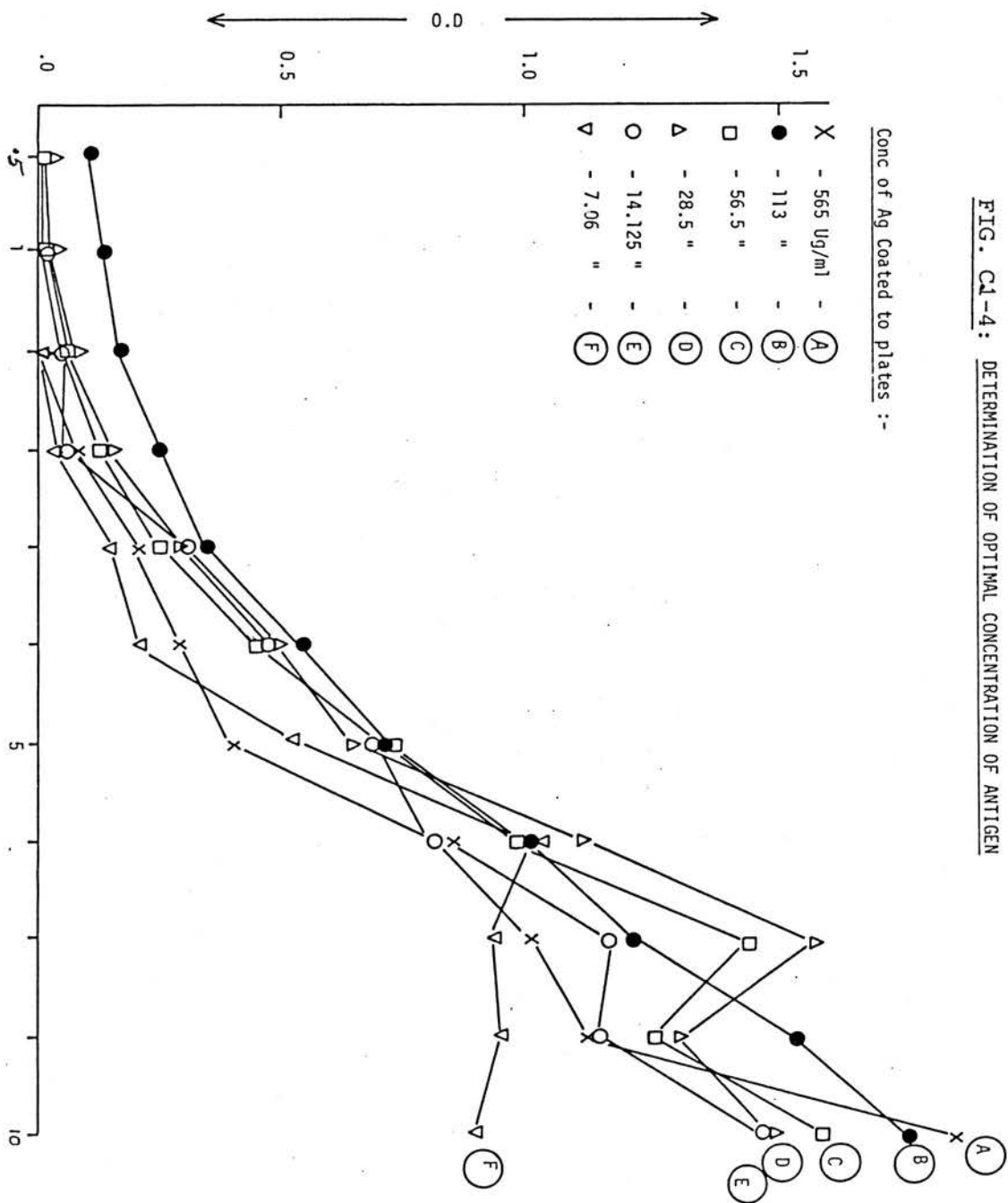
TABLE C.1-3

Results of antitetanus standard assayed against different concentrations of Tetanus Toxoid

I.U./ml	(O.D.) with different concentrations of Tetanus Toxoid					
Antitetanus IgG (P.F.C.)	565 $\mu\text{g/ml}$	113 $\mu\text{g/ml}$	56.5 $\mu\text{g/ml}$	28.5 $\mu\text{g/ml}$	14.125 $\mu\text{g/ml}$	7.06 $\mu\text{g/ml}$
0.5	1.834	1.77	1.589	1.498	1.491	0.889
0.25	1.122	1.558	1.252	1.291	1.27	0.936
0.125	1.086	1.311	1.372	1.597	1.171	0.933
0.06	0.835	1.062	0.967	1.109	0.79	1.069
0.03	0.401	0.705	0.703	0.651	0.757	0.532
0.016	0.289	0.536	0.463	0.488	0.445	0.224
0.008	0.208	0.345	0.256	0.302	0.26	0.162
0.004	0.084	0.237	0.142	0.153	0.061	0.064
0.002	0.014	0.169	0.069	0.069	0.057	0.016
0.001	0.0	0.129	0.033	0.037	0.015	0.0
0.0005	0.0	0.104	0.011	0.011	0.0	0.0

Note that within the range of antigen concentration of 28.5-113  $\mu\text{g/ml}$ , antibody level as low as  $(5 \times 10^{-4})$  I.U./ml can be detected.

FIG. C1-4: DETERMINATION OF OPTIMAL CONCENTRATION OF ANTIGEN



This ELISA assay appeared suitable over a wider range of antigen concentrations, a concentration of 50 µg/ml of tetanus toxoid was selected for routine use.

TABLE C.1-4

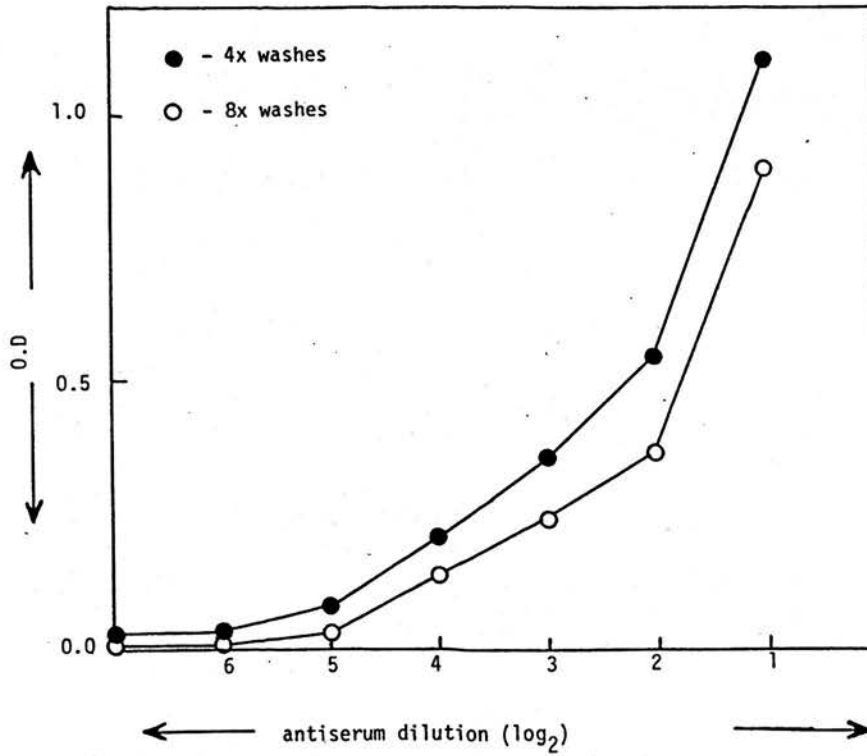
Effect of excess washing on ELISA

Tetanus IgG (P.F.C.) I.U./ml	A405	
	Washed 2 & 4 times	Washed 4 & 8 times
0.5	1.07	0.9
0.25	0.54	0.37
0.125	0.4	0.25
0.06	0.21	0.16
0.03	0.08	0.03
0.016	0.03	0.01
0.008	0.03	0.01

The absorbance at 405 n.m. of the standard samples decreases as the number of washes are increased (see also Fig.5).

FIG. C.1-5

EFFECT OF EXCESS WASHING ON THE TEST RESULTS



The drop in O.D. ( $A_{405}$ ) values of the anti-tetanus serum dilutions indicate that some protein can be detached from wells of the ELISA plate by excess washing.

TABLE C.1-5

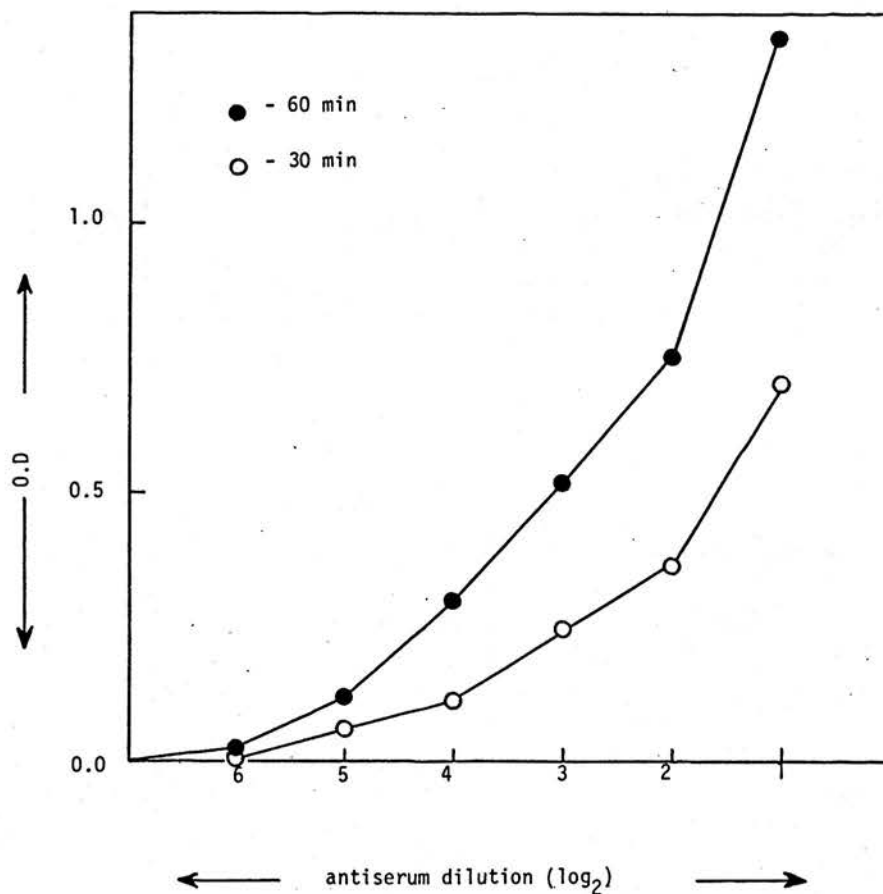
Effect of time of incubation  
with substrate at 37°C

Tetanus- IgG P.F.C. I.U.	A405	
	60 min	30 min
0.5	1.36	0.69
0.25	0.75	0.37
0.125	0.52	0.25
0.06	0.3	0.11
0.031	0.12	0.06
0.016	0.02	0.02
0.008	0.01	0.01

There is a twofold increase in absorbance at 405 n.m. of the test samples when the incubation time with substrate is doubled (see also Fig.6).

FIG. C.1-6

EFFECT OF INCUBATION PERIOD WITH SUBSTRATE AT 37°C



One hour incubation of ELISA plates was chosen for routine use, since it gives good sensitivity. Shorter incubation time can be used providing a standard is used in each assay, and the test results are evaluated accordingly.

TABLE C.1-6

EFFECT OF DIFFERENT CONCENTRATIONS OF CONJUGATE TESTED  
ON DIFFERENT ANTITETANUS STANDARDS

Standards	Antibody Concentration I.U./ml	O.D. Dilution of Conjugate		
		1:500 (O.D.)	1:800 (O.D.)	1:1000 (O.D.)
Tetanus - IgG (P.F.C.)  (a)	1.0	2.0	1.748	1.157
	0.1	2.0	1.193	0.94
	0.05	1.809	1.238	9.761
	0.01	1.084	0.462	0.453
	0.005	0.633	0.421	0.206
	0.001	0.047	0.0	0.0
	0.0005	0.0	0.0	0.0
Tetanus - IgG - (Humotet)  (b)	1.0	2.0	1.497	1.261
	0.1	1.838	0.981	0.966
	0.05	1.371	1.123	0.746
	0.01	0.646	0.240	0.322
	0.005	0.310	0.151	0.221
	0.001	0.0	0.0	0.0
	0.0005	0.0	0.0	0.0
Antitetanus serum (NIBSAC)  (c)	1.0	2.0	1.618	1.223
	0.1	1.818	0.95	0.937
	0.05	1.538	0.95	0.853
	0.01	0.764	0.324	0.333
	0.005	0.346	0.255	0.253
	0.001	0.0	0.0	0.0
	0.0005	0.0	0.0	0.0

Conjugate dilution of 1:500 gave the highest sensitivity in all 3 different anti-tetanus standards used (see also Fig.C.1-7, a,b,c & d).

FIG. C.1-7

The optimal concentration of conjugate was determined by testing different dilutions of conjugate against 3 different tetanus antibody standards as follows:-

- (a) Tetanus IgG (P.F.C.)
- (b) Tetanus IgG (Humotet)
- (c) Tetanus antiserum (NIBSAC)
- (d) All 3 standards tested using optimum concentration of conjugate (1:500)



FIG. C.1-7

EFFECT OF DIFFERENT CONCENTRATION OF CONJUGATE TESTED  
WITH 3 DIFFERENT ANTITETANUS STANDARDS.

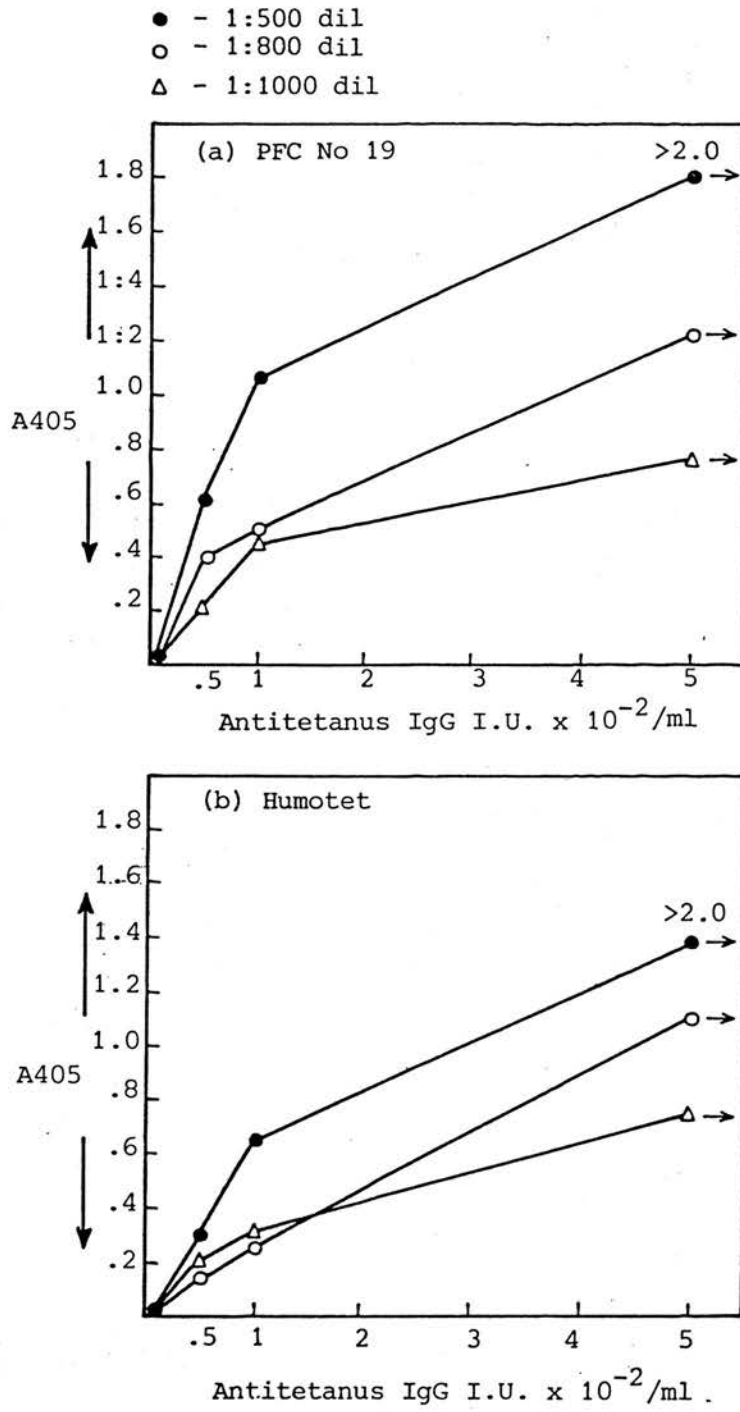


FIG. C.1-7 contd.

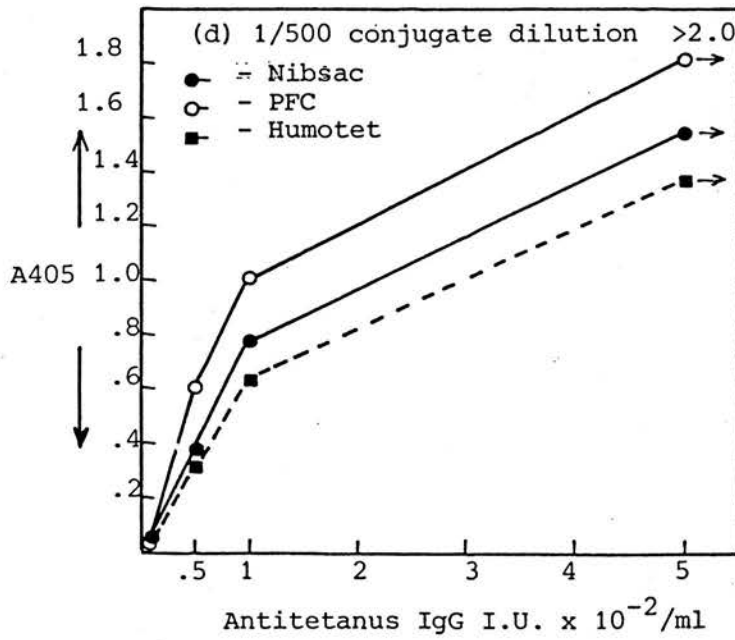
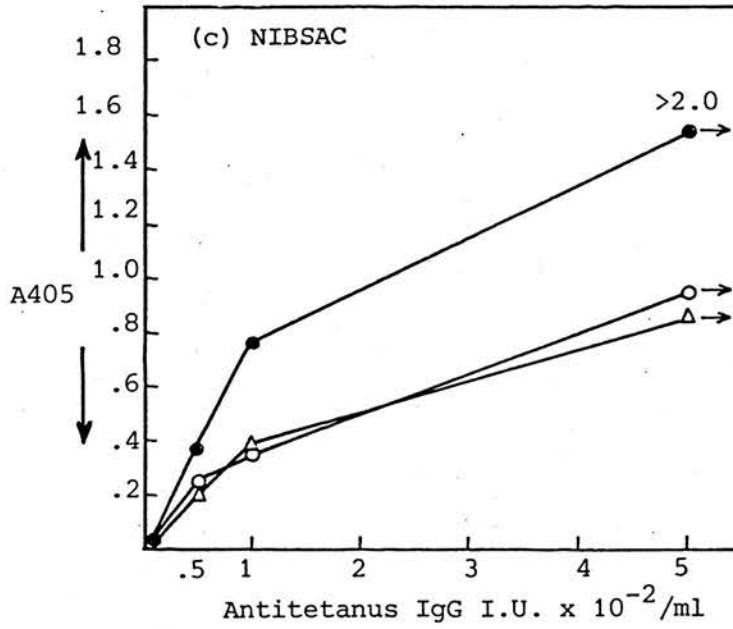


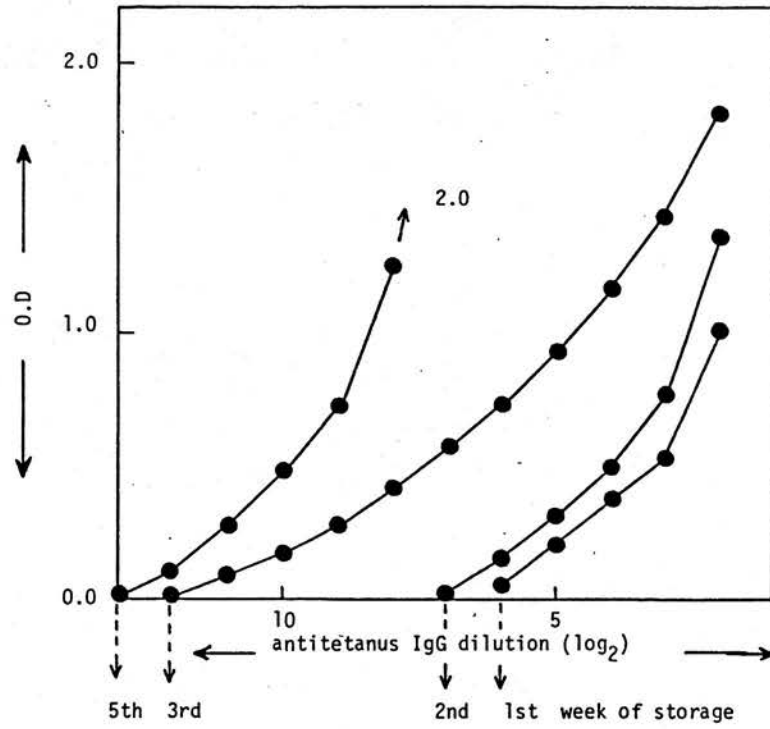
TABLE C.1-7  
SHELF LIFE DETERMINATION OF PLATE COATED AND STORED AT 4°C  
WITH TETANUS TOXOID SOLUTION IN THE WELLS

Tetanus IgG (P.F.C.) I.U./ml	O.D. at further storage time			
	1st Week	2nd Week	3rd Week	5th Week
0.5	1.07	1.38	1.81	2.0
0.25	0.54	0.75	1.41	2.0
0.125	0.41	0.52	1.16	2.0
0.06	0.21	0.3	0.92	2.0
0.03	0.08	0.12	0.74	2.0
0.016	0.03	0.02	0.62	2.0
0.008	0.03	0.01	0.41	1.24
0.004	0.0	0.0	0.28	0.73
0.002	0.0	0.0	0.16	0.48
0.001	0.0	0.0	0.08	0.31
0.0005	0.0	0.0	0.0	0.12
0.00025	0.0	0.0	0.0	0.0

The O.D. has increased with every week of additional incubation. In the 5th week the O.D. was above the readable range ( 2.0) of the multiscan (see also Fig.8).

FIG. C.1-8

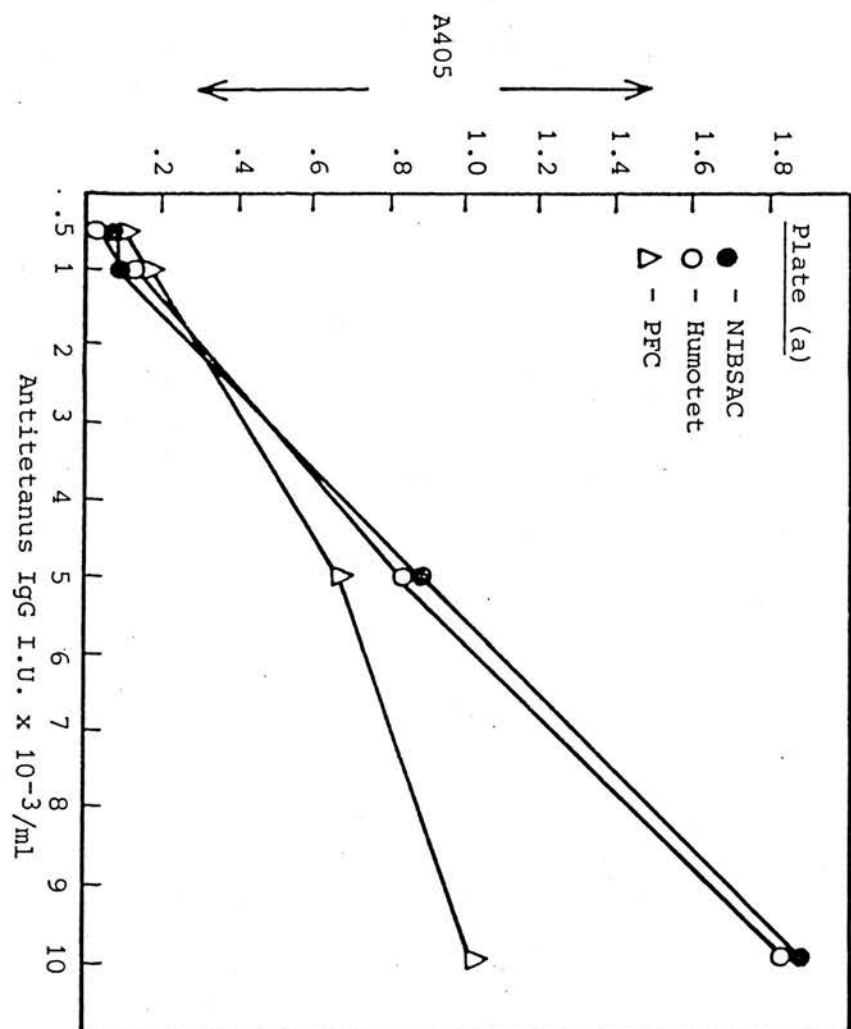
SHELF LIFE DETERMINATION OF PLATE COATED AND STORED  
AT 4°C WITH TETANUS TOXOID SOLUTION IN WELLS



The sensitivity of the test increased greatly with storage of the Ag-coated plates, possibly due to further concentration and absorption of the tetanus toxoid by the solid phase.

FIG. C.1-9 (a): RESULTS OF THREE DIFFERENT ANTITETANUS STANDARDS ASSAYED IN PLATES COATED AND STORED IN THREE DIFFERENT WAYS

Plate coated and stored at 4°C with tetanus toxoid solution in the wells 25 days prior to assay



N.B. O.D. increases above the acceptable range (unreadable by Multiscan) may be due to evaporation and therefore further concentration of Ag.

FIG. C.1-9 (b) : PLATE COATED AND INCUBATED OVERNIGHT AT 4°C WITH  
Ag SOLUTION IN WELLS AND USING THE NEXT DAY

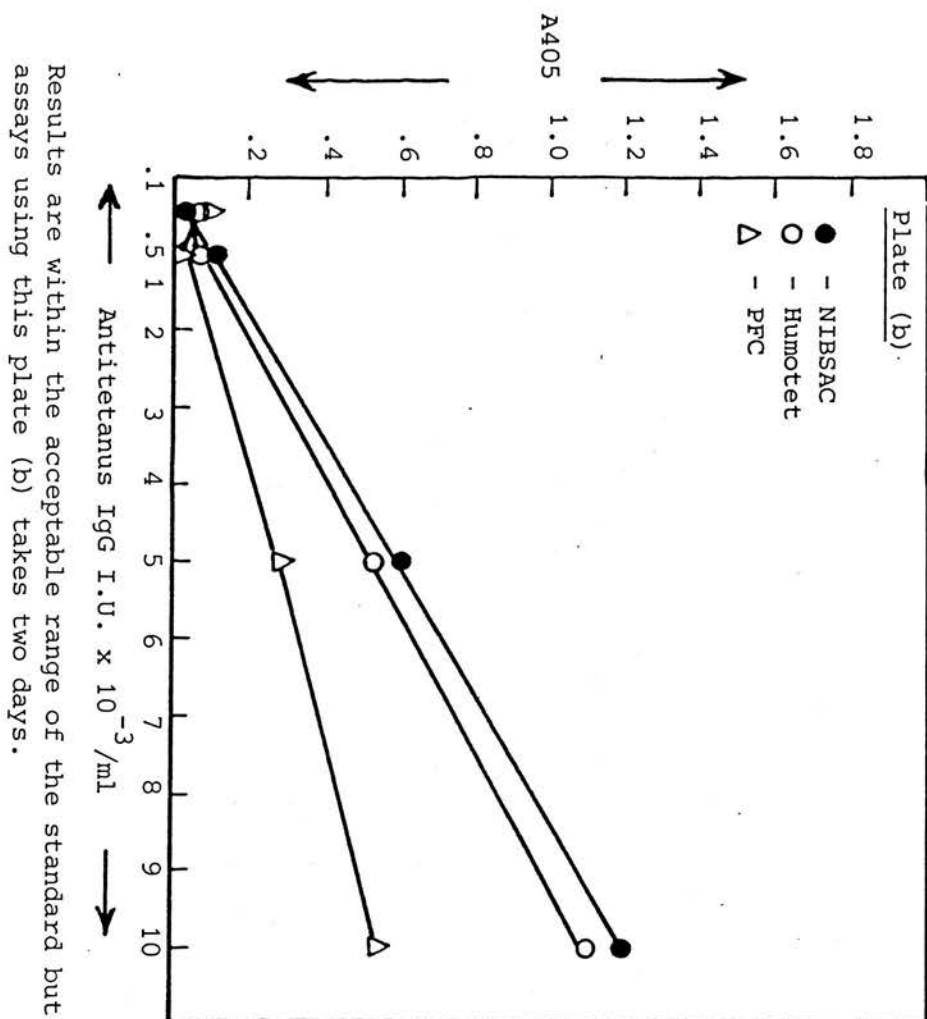
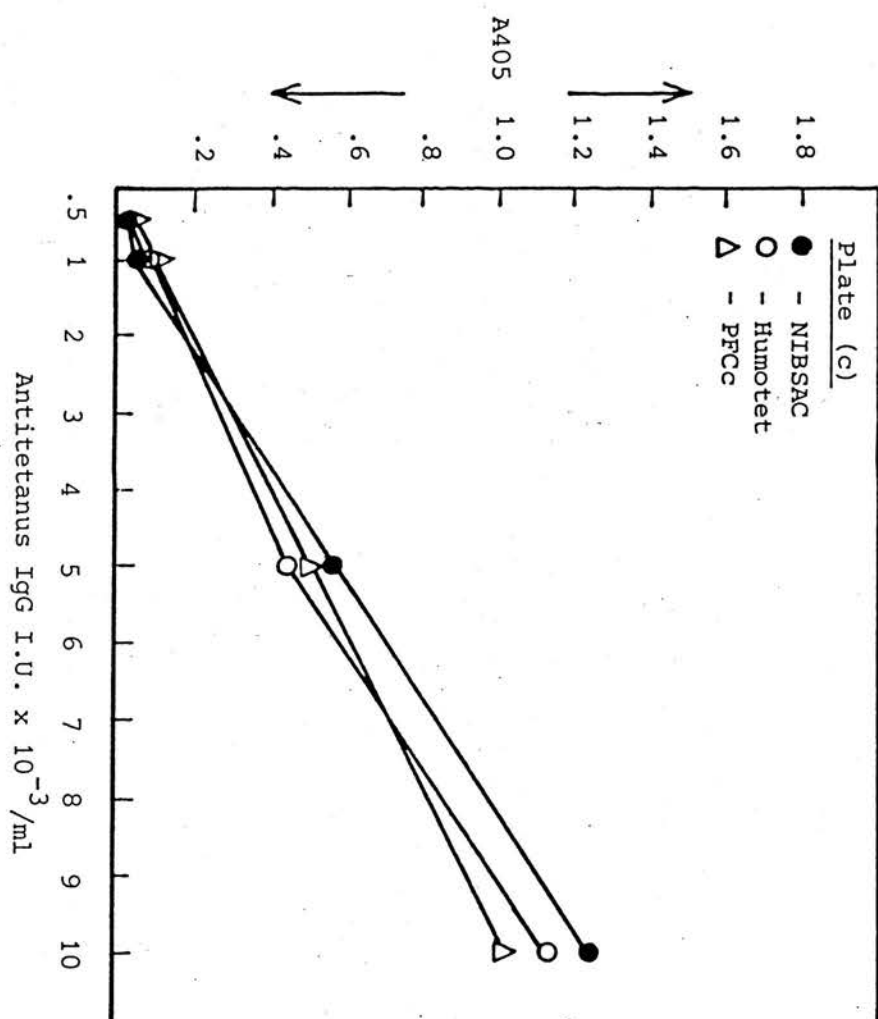


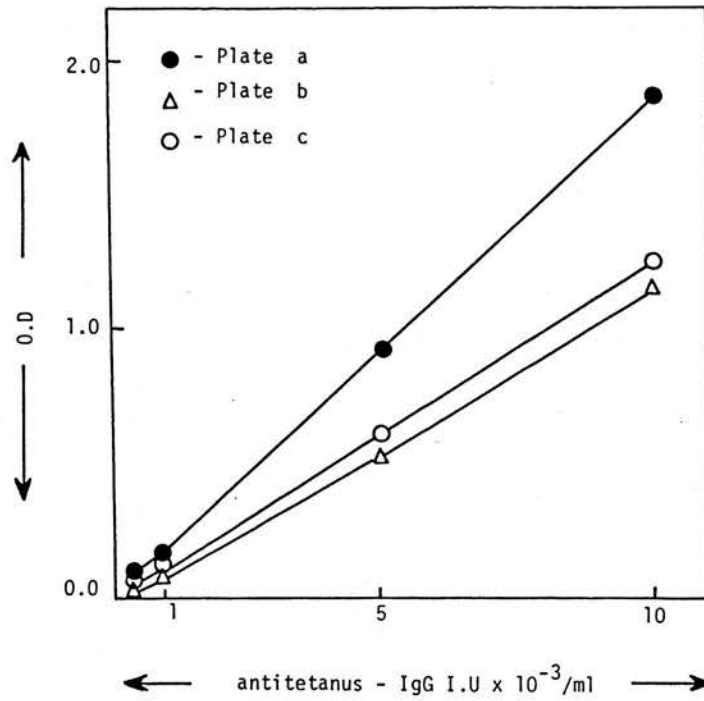
FIG. C.1-9 (c) : PLATE COATED, STORED WITH Ag SOLUTION OVERNIGHT AT 4°C, WASHED AND DRIED AND STORED.



N.B. with this coating procedure all 3 different standards give similar results (same conc.std. (I.U.) used).

FIG. C.1-9 (d)

EFFECT OF DIFFERENT STORAGE AND COATING PROCEDURES



Anti-tetanus standard (NIBSAC) was tested in plates which were coated and stored under different conditions. Plate (c) was chosen for long time storage since the results given were within the acceptable range (O.D. < 2) and reproducible. Plates coated and stored in this manner had been found to have a shelf life of at least 1 year at 4°C (See also Table 8).



TABLE C.1-8

QUANTITATIVE MEASUREMENT OF ANTITETANUS ANTIBODY IN SERA OF TETANUS IMMUNISED DONORS USING PLATES  
COATED AND STORED BY 3 DIFFERENT PROCEDURES AND READ AGAINST 3 DIFFERENT ANTITETANUS STANDARDS IN EACH PLATE

Samples (Immunized donors)	Plate (a)			Plate (b)			Plate (c)		
	NIBSAC	HUMOTET	P.F.C.	NIBSAC	HUMOTET	P.F.C.	NIBSAC	HUMOTET	P.F.C.
1	30	30	34	37	40	70	18	31	28
2	41	43	55	50	54	>	22	24	19
3	39	40	50	44	49	95	57	64	62
4	31	31	36	42	46	90	77	85	91
5	70	72	>	68	73	>	80	89	95
6	69	70	>	66	70	>	81	90	95
7	18	19	19	28	31	52	28	31	28
8	75	77	>	36	40	75	28	28	31
Standards	15	250	180	15	250	180	15	280	180

N.B. Samples were tested at single dilution of 1:10,000  
> = above the acceptable range of standard

TABLE C.1-9

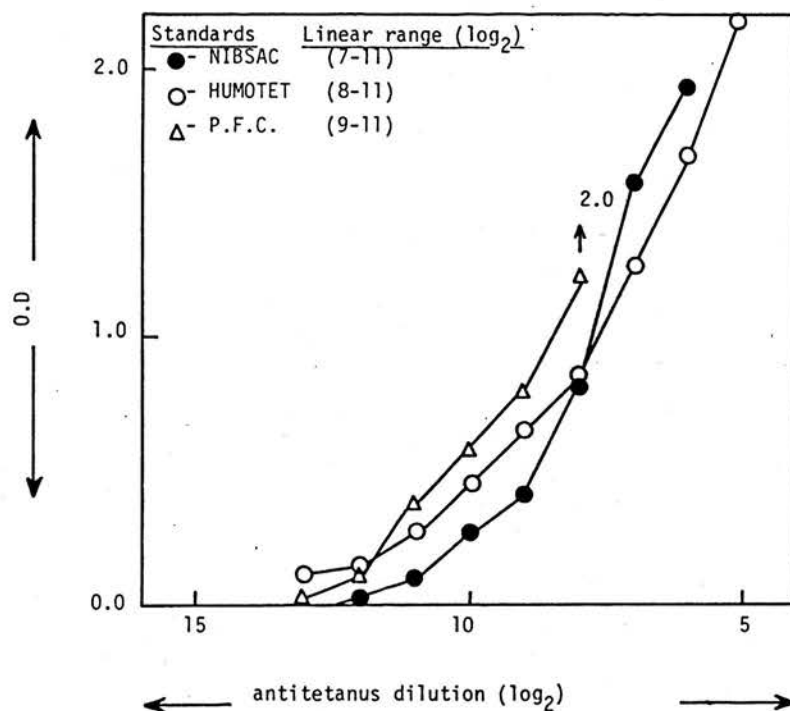
O.D. OF DIFFERENT STUDIES ON PLATES COATED AND STORED BY 3 DIFFERENT METHODS

Antitetanus Standard Concentration (I.U./ml)	Plate (a)			Plate (b)			Plate (c)		
	NIBSAC	HUMOTET	P.F.C.	NIBSAC	HUMOTET	P.F.C.	NIBSAC	HUMOTET	P.F.C.
1.0	> 2.0	> 2.0	> 2.0	> 2.0	> 2.0	> 2.0	> 2.0	> 2.0	> 2.0
0.5	> 2.0	> 2.0	> 2.0	> 2.0	1.67	1.83	1.91	1.79	> 2.0
0.1	> 2.0	> 2.0	> 2.0	1.80	1.62	1.59	1.78	1.58	> 2.0
0.05	2.0	2.0	2.0	1.54	1.36	1.42	1.49	1.34	1.56
0.01	1.85	1.86	1.05	1.17	1.09	0.52	1.24	1.14	1.01
0.005	0.89	0.89	0.65	0.56	0.54	0.26	0.56	0.45	0.47
0.001	0.08	0.15	0.14	0.11	0.11	0.08	0.05	0.08	0.11
0.0005	0.067	0.012	0.09	0.04	0.04	0.006	0.0	0.002	0.02
Acceptable Range (Linear)	0.08- 1.8	0.1- 1.8	0.1- 1.0	0.04- 1.2	0.04- 1.1	0.08- 0.52	0.05- 1.2	0.08- 1.1	0.1- 1.0

> - Above the acceptable range which the multiscan can measure. Note that anti-tetanus standards tested in plate (c) gave a wider linear range than they did in plate (a) and (b).

FIG. C.1-10

COMPARISON OF DIFFERENT ANTITETANUS STANDARDS



It is apparent that the anti-tetanus serum standard (NIBSAC) is the most suitable for the routine assay, and its linear working range is higher than the other two standards (see also Table 9).

TABLE C.1-10

RESULTS OF ANTITETANUS STANDARD DILUTIONS (NIBSAC) READ AGAINST

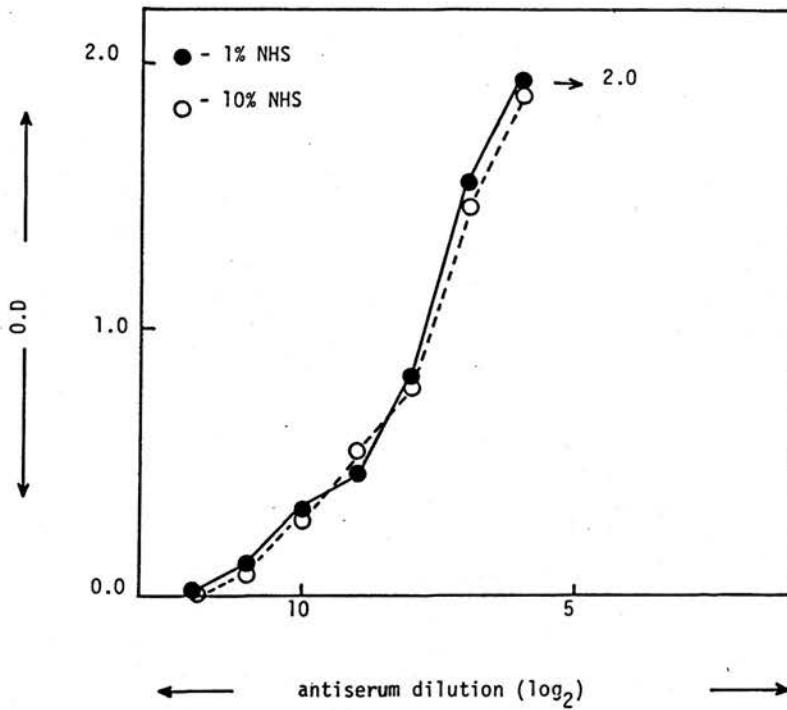
1% and 10% NHS. BOTH RESULTS VERY SIMILAR

Tetanus-Antiserum (NIBSAC) I.U./ml	O.D.	
	1% NHS	10% NHS
1.0	> 2.0	> 2.0
0.1	1.63	1.57
0.05	1.25	1.15
0.01	0.94	0.87
0.005	0.51	0.46
0.001	0.10	0.05
Twofold dilution		
0.5	> 2.0	> 2.0
0.25	> 2.0	> 2.0
0.125	> 2.0	> 2.0
0.06	> 2.0	> 2.0
0.03	1.91	1.88
0.016	1.50	1.46
0.008	0.81	0.78
0.004	0.45	0.55
0.002	0.33	0.32
0.001	0.12	0.11

Both concentrations of NHS were negative by ELISA. The multiscan plate-reader was blanked using 1% and then 10% NHS, and antiserum dilutions were read against each. Similar results were obtained indicating that there is no non-specific binding (see also Fig.11).

FIG. C.1-11

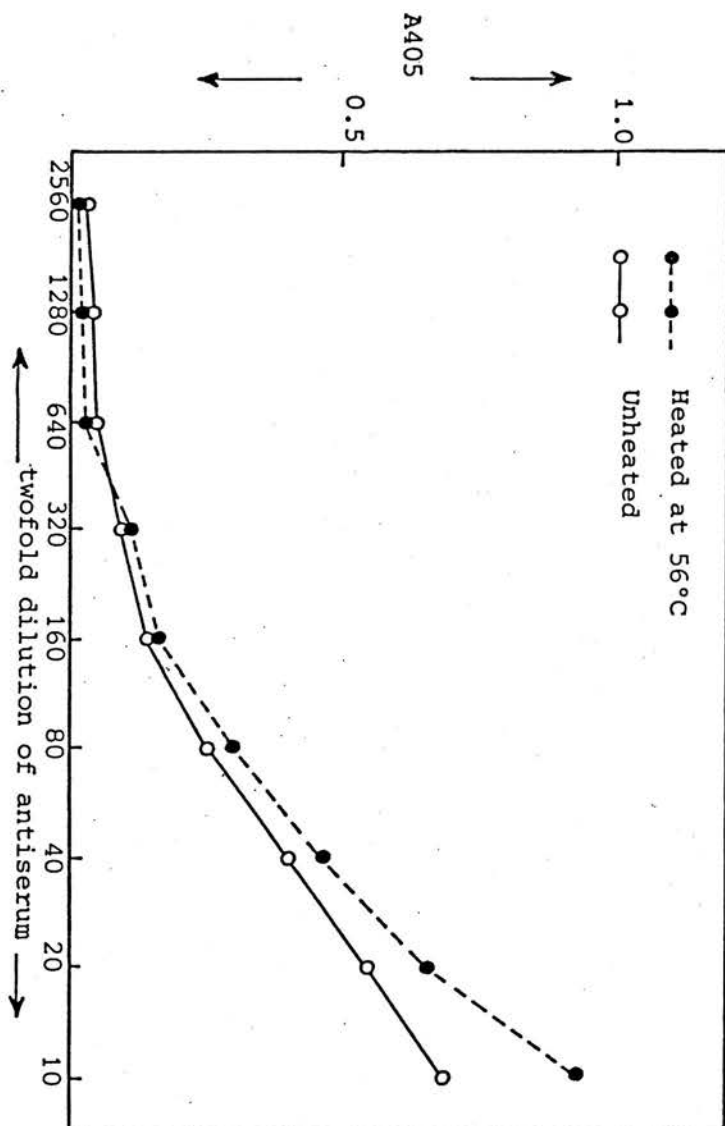
DETERMINATION OF NON-SPECIFIC BINDING BY READING  
ANTITETANUS STANDARD AGAINST NORMAL HUMAN SERUM (NHS)



The multiscan ELISA-plate reader was blanked using 1% and 10% NHS, and then the test samples were read against each concentration of NHS. There were no differences between the two test results, indicating that there is no non-specific binding using this technique.

FIG. C.1-12

EFFECT OF HEAT INACTIVATION OF SERUM IN ANTI-TETANUS  
ANTIBODY TITRES DETERMINED BY ELISA



A more linear curve, and an increase in reaction at higher concentrations were observed when the donor's plasma was pre-heated. This phenomenon is thought to be caused by heat-labile serum component.

TABLE C.1-11

DETERMINATION OF OPTIMAL CONCENTRATION OF SHEEP ANTI-MOUSE Ig  
(AP-CONJUGATED) AND OF NON-SPECIFIC BINDING

Conjugate dilution	Anti-tetanus serum control	ELISA-titre of antibody
1:50	Pos. Control Neg. Control	1:1024,00 1:100
1:100	Pos. Control Neg. Control	1:25,600 1:50
1:500	Pos. Control Neg. Control	1:51,200 0

Dilution of 1:500 for conjugate was selected for routine mouse assay, since it shows optimum specificity with good sensitivity.

C.1.4

DEVELOPMENT OF AN ASSAY TO MEASURE ANTI-TETANUS ANTIBODIES  
OF DIFFERENT MOUSE Ig-ISOTYPES (SEI)



#### C.1.4 Development of an assay to measure anti-tetanus antibodies of different mouse Ig-isotypes (SEI)

##### 1.4.1 Introduction

Serum immunoglobulins (Ig) have been divided into different classes on the basis of a variety of properties including the presence of non cross-reacting antigenic determinants found on the Fc fragment. These antigenic differences reflect amino acid differences in the constant portion of the heavy chains. Five classes of Igs have been so defined, some of which have been further divided into subclasses according to more minor Fc differences (Spiegelberg, 1974). The Ig-subclasses differ in their physical, chemical and biological properties (Feinstein et al. 1964, Micuson, 1975). More recently the term isotype has been used to cover both class and subclass differences between various immunoglobulins (Ig).

In the mouse the major Ig-isotypes IgM, IgA, IgG<sub>1</sub> and IgG<sub>2</sub> were first identified by Fahey et al. (1964a). These workers subsequently showed that the mouse IgG<sub>2</sub> existed in two isotypic forms, designated IgG<sub>2a</sub> and IgG<sub>2b</sub> (Fahey et al., 1964b). Later IgG<sub>3</sub> was discovered by Grey et al. (1971).

The ready availability of commercial antisera to the mouse Ig-isotypes, and the lack of similar reagents to human isotypes at the time this work was commenced, made the mouse a suitable model to study the Ig-isotypic responses following tetanus injection.

The mouse isotypes are similar in many regards to the human (Stanworth et al., 1973). It was hoped that this study would provide useful information with regard to projected investigations in human with the ultimate aim of discovering the importance of particular human Ig-isotype in the response to infection and in passive Ig therapy.

Other workers have measured total Ig-isotype levels in mice by radial immuno-diffusion (Yount et al., 1970; Van der Giesen, 1975; Shakib et al., 1975), immuno-diffusions (Stanislowski, 1976; Kronvall et al., 1970), and radioimmunoassay (Morell et al., 1972; Radl et al., 1975).

Specific isotype-associated antibody levels to tetanus toxoid can be measured by immunodiffusion (Ourth et al., 1977), immunofluorescence methods (Van der Giessen, 1976) and by a modified RIA (Carrel et al., 1972). Such procedures with the exception of RIA are however semiquantitative. RIA, although sensitive, requires use of rather large quantities of anti-isotype reagents, with limited shelf life of any of the iodinated reagents, long incubation periods, and has hazards associated with the handling of radioisotopes (Ekins, 1980).

Enzyme immunoassays have certain advantages over conventional RIA in that they are as sensitive, are more rapid and less expensive. A Sandwich Enzyme Immunoassay (SEI) was therefore developed to quantify the level of antibodies of a particular Ig-isotype in mice. This was based on the ELISA technique described earlier (Section C.1).

#### 1.4.2 Materials and Methods

Positive control: pooled anti-tetanus mouse serum obtained by boosting preimmunized mice (ip) with 10  $\mu$ g of tetanus toxoid. The mice were bled seven days later. The sera were pooled, aliquoted and stored at

-20°C. The same pool was used as a positive control for mouse anti-tetanus antibody measurement in other studies.

Negative control: pooled normal mouse BALB/c serum (NMS).

Conjugate: Alkaline phosphatase (AP) conjugated goat anti-rabbit Ig, at 1:2000 dilution (see list of antisera).

The above materials are those which differ from materials used in the direct ELISA (Section C.1).

The procedure of SEI assay was essentially similar to the direct ELISA as previously described (Section C.1), except that it incorporated an additional step (Fig.1-Step 2). This involved addition of what might be called bridging antibody (antibody specific to mouse Ig-isotypes) to the Ag-Ab complex already fixed on the plate (See Fig.1). The optimal concentration of the bridging antibody had to be determined. The binding of the bridging antibody was detected by the addition of AP-conjugated anti-rabbit Ig (H & L), followed by substrate. Experimental antisera were initially diluted 1:10 or 1:100 followed by two-fold serial dilutions in V/V) 1% BSA in PBS. The dilution of the experimental anti-tetanus serum was 1:200. This constant dilution was initially used in order to optimize the concentration of the bridging antibody.

The absorbance at 405 nm (corrected for background) was determined in a Multiskan microtitre plate reader. The OD<sub>405</sub> values were plotted against log<sub>2</sub> of the antibody titres.

### 1.4.3 Results and Discussion

#### 1.4.3.1 Determination of the optimal dilution of bridging antibodies (antibody specific to Ig-isotype) for SEI

Initially antisera specific to each Ig-isotypes were tested against constant concentration of primary antibody (pooled anti-tetanus mouse serum) using tetanus toxoid coated plates. The dilution of each bridging antibody providing a broad range in the linear portion and giving  $OD_{405} = 1.0$  was selected. Fig. 2 (A-F) illustrates SEI plots for each isotype specific antibody, and a defined plateau in the region of antigen saturation (Ag-Ab complex). The selected dilution for each isotype specific antibody was divided by 3 in order to ensure antibody would be in excess quantity, and to increase sensitivity. These dilutions of the bridging antibody finally selected are listed in Table 1 and were used in routine assay.

Prior to determining the optimum dilutions of anti-isotype antibody preliminary experiments were performed with an antibody to total mouse Ig. The initial experiment provided a basis for the subsequent experiments with the more expensive isotype specific reagents (Fig. 2(G)).

#### 1.4.3.2 Determination of non-specific binding

As a negative control a pooled normal mouse serum (NMS) was always assayed in parallel with the pooled antitetanus mouse serum. Fig. 2(G) and Fig.3 indicate no non-specific binding by NMS but high concentration of IgG<sub>1</sub> antibody was detected in the antitetanus serum. The IgG<sub>1</sub> antibody level decreases linearly with antiserum dilution, and is still detectable in the sample at a dilution of 1:100,000 (Fig.3). The results were corrected for background using 1% BSA. Both these positive and negative controls were included routinely in all

assays for individual Ig-isotype measurement. During the course of the study we found natural occurring antibodies to tetanus of the IgM and IgA isotypes. These will be discussed later.

#### 1.4.3.3 SEI assay using optimized dilution of Ig-isotype specific antibody

The optimum dilution of each bridging antibody (see Table 1) was incubated with various dilutions of antitetanus sample on tetanus coated plates. After washing off the unbound antibody the conjugate and substrate were added as before. Fig.4 illustrates the levels of anti-tetanus antibody of each isotype in pooled mouse antisera used to standardize the Sandwich Enzyme Immunoassay. It should be stressed that every batch of isotype specific antibody should be titrated as above prior to its use in a routine assay.

#### 1.4.3.4 Comparison in the distribution of tetanus antibody isotypes of Protein A-Sepharose fractions with SEI technique

Mouse anti-tetanus serum was fractionated on protein A-sepharose-4B according to the method described in Section B.5, and by Ey et al. (1978). The eluted fractions (Fig.5) of each peak were pooled and concentrated. The amount of isotype specific antibody to tetanus was measured by SEI (Fig.6). These results support the specificity of the anti-isotype antisera used. There is however IgA carry over in fraction 3 and 4 (Fig.6). This could be due to small amounts of anti-IgG<sub>1</sub> and IgG<sub>2a</sub> activity in the IgA region or incomplete resolution of these molecules by protein A affinity chromatography. It should be noted that although separation of IgG<sub>3</sub> isotype has not been included in the protein A separation technique used (Ey et al., 1978). Small amounts of this isotype were detected by the SEI assay.

#### 1.4.4 Conclusions

1.4.4.1 The SEI assay developed to measure anti-tetanus antibodies of different classes and subclasses (isotypes), is rapid, sensitive and specific.

1.4.4.2 Because the isotype specific antisera can be used at high dilutions it is sparing on expensive reagents.

1.4.4.3 The specificity of the anti-isotype antisera used is supported by the distribution of the antibodies by Protein A-Sepharose fractions.

1.4.4.4 It is widely applicable. It can measure Ig-isotype levels in any antiserum, as long as the relevant antigen can bind to the solid phase. It should also be noted that antisera to Ig-isotypes of many species are commercially available.

1.4.4.5 The SEI (and ELISA) assay should be readily adaptable to automation and computerized data processing.

1.4.4.6 It should prove particularly useful in determining the isotype of monoclonal antibodies.

FIG. C.1.4-1

SANDWICH ENZYME IMMUNOASSAY (SEI)  
FOR DETECTION OF Ig-ISOTYPES

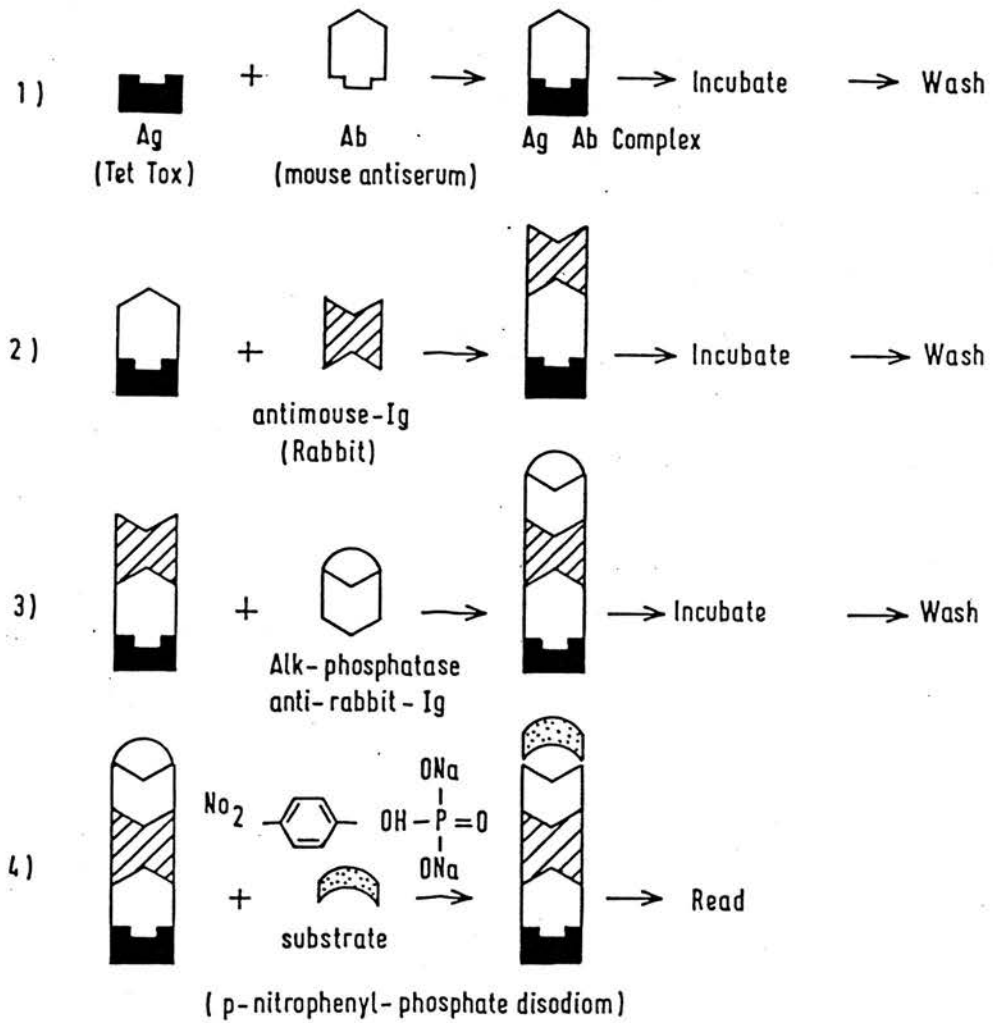


FIG. C.1.4-2 (A & B) : DETERMINATION OF OPTIMAL DILUTION OF ANTIMOUSE Ig-ISOTYPES  
 Twofold dilutions of (Rabbit) anti-mouse-Ig<sub>1</sub> (A) , and -Ig<sub>3</sub> (B) were assayed by  
 SEI against fixed dilution (1:200) of mouse anti-tetanus serum.

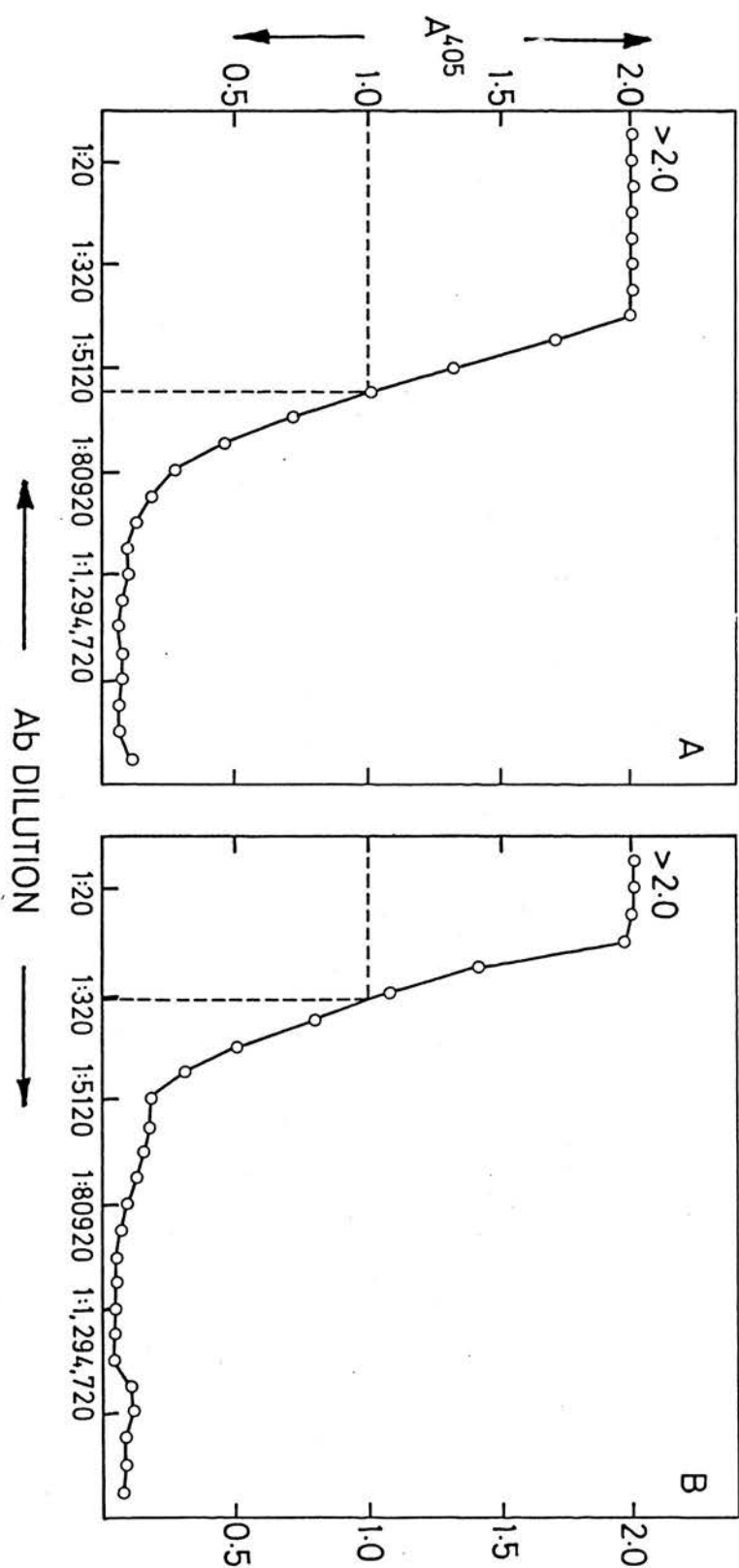




FIG. C.1.4-2 (C & D) : DETERMINATION OF OPTIMAL DILUTION OF ANTIMOUSE Ig-ISOTYPES

Twofold dilutions of (Rabbit) antimouse-IgG<sub>2a</sub> (C) and -IgG<sub>2b</sub> (D) were assayed by SEI against fixed dilution (1:200) of mouse anti-tetanus serum.

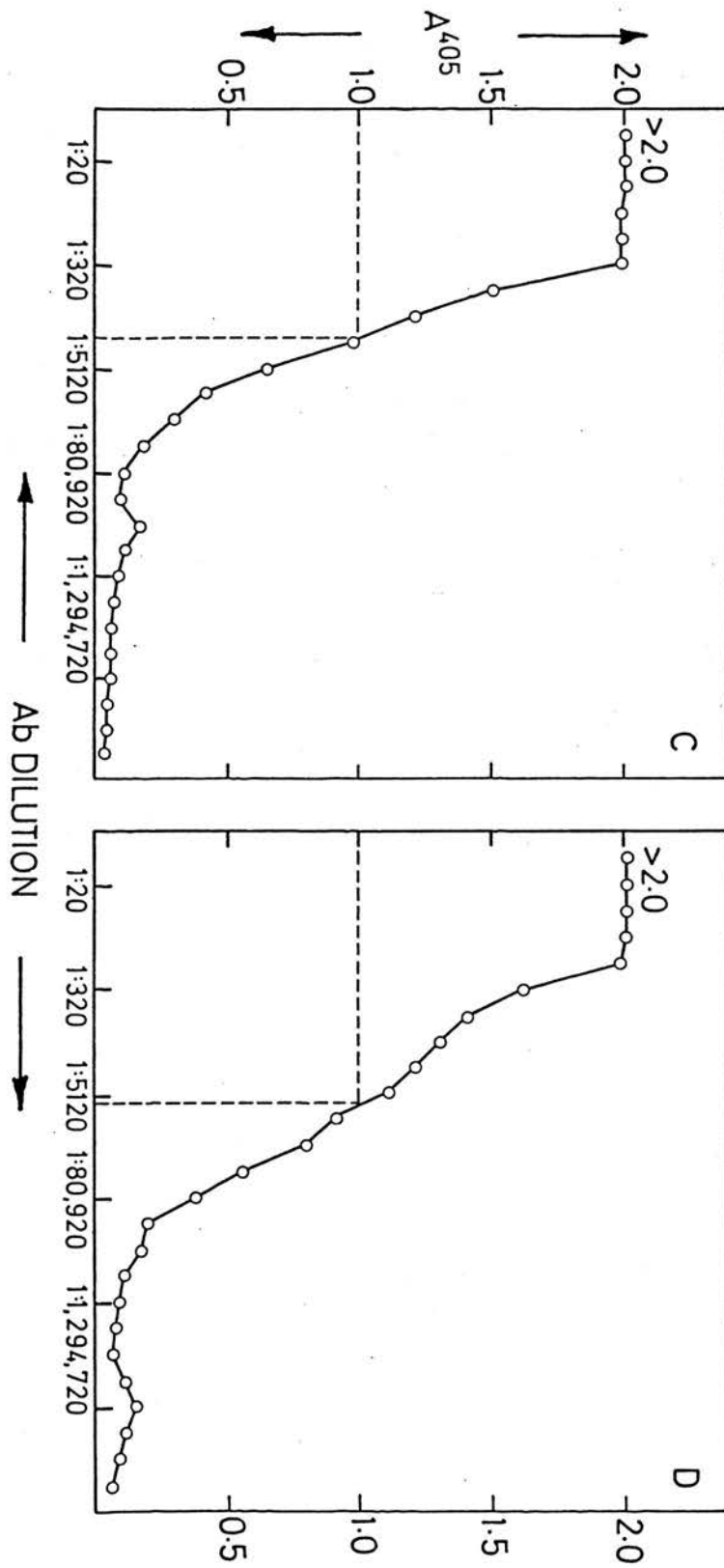


FIG. C.1.4-2 (E & F): DETERMINATION OF OPTIMAL DILUTION OF ANTIMOUSE Ig-ISOTYPES  
 Twofold dilutions of (Rabbit) antimouse-IgA (E) and -IgM (F) were assayed by SEI  
 against fixed dilution (1:200) of mouse anti-tetanus serum

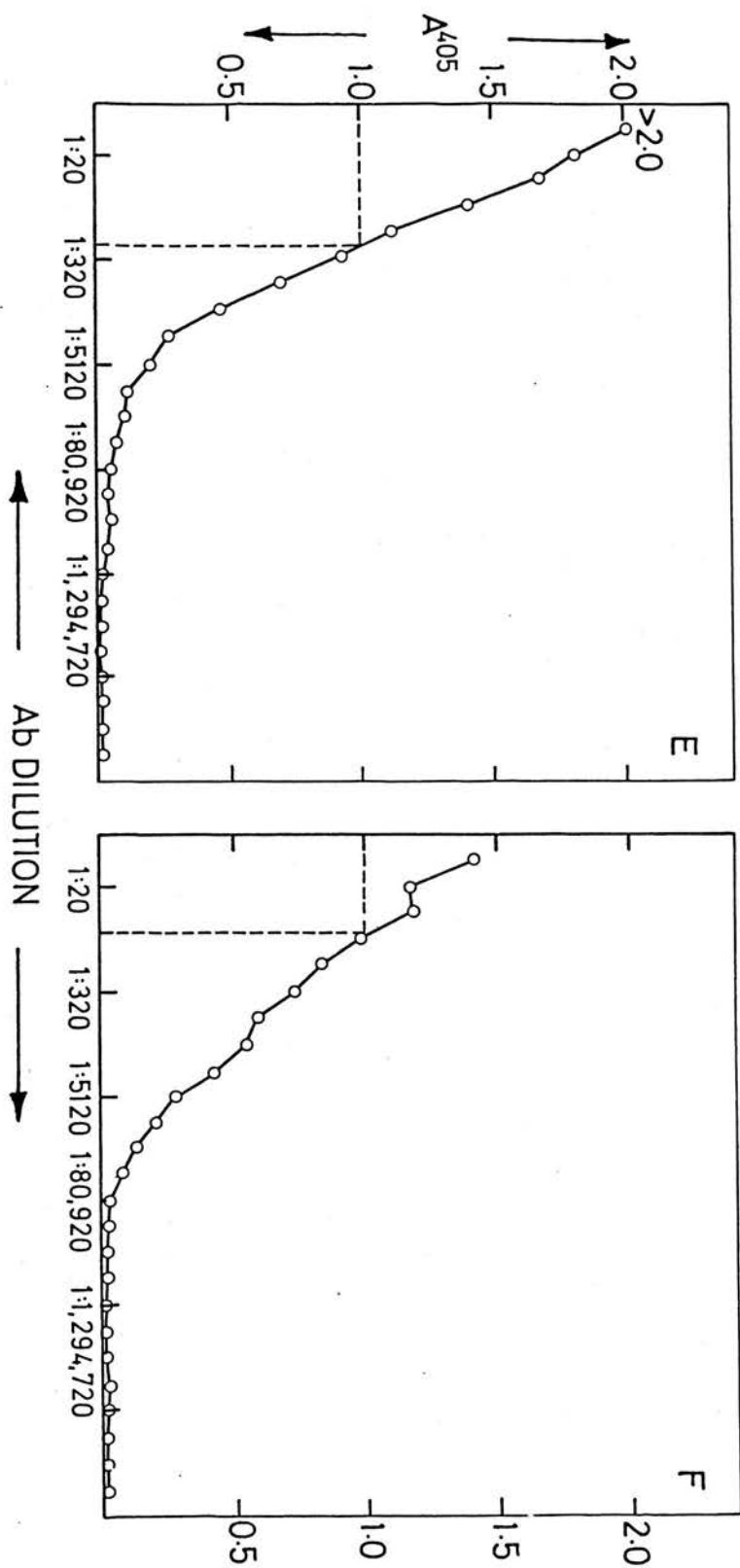
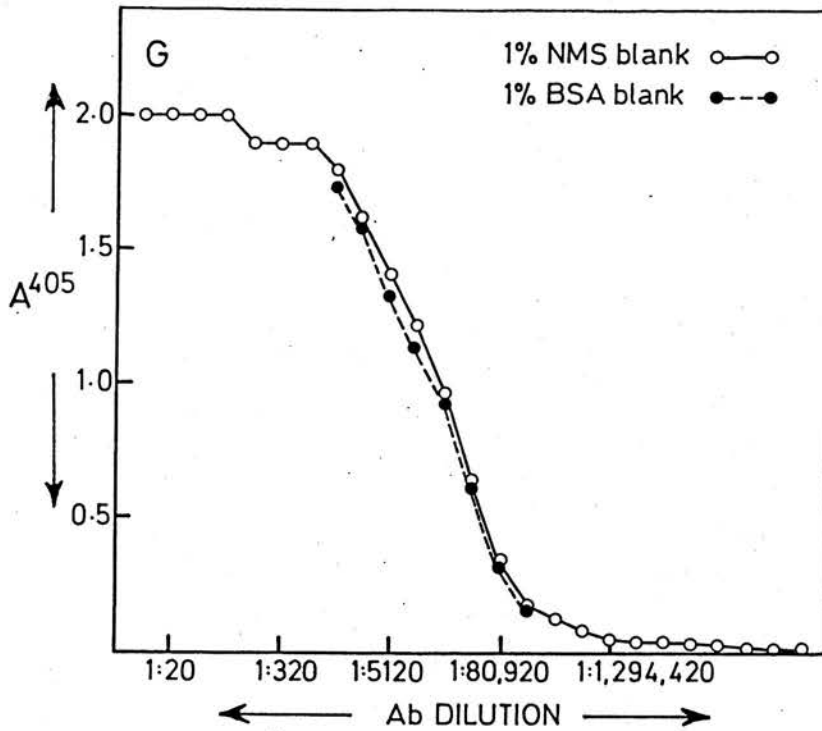


FIG. C.1.4-2 (G): PRELIMINARY EXPERIMENT FOR SEI  
PERFORMED WITH ANTIBODY TO TOTAL MOUSE-Ig



Twofold dilutions of (Rabbit) antimouse-Ig were tested against fixed concentration (1:200) of mouse anti-tetanus serum. Values were plotted after corrections for background using both 1% NMS and 1% BSA as negative controls. This Fig. also demonstrates the high specificity of the SEI assay.

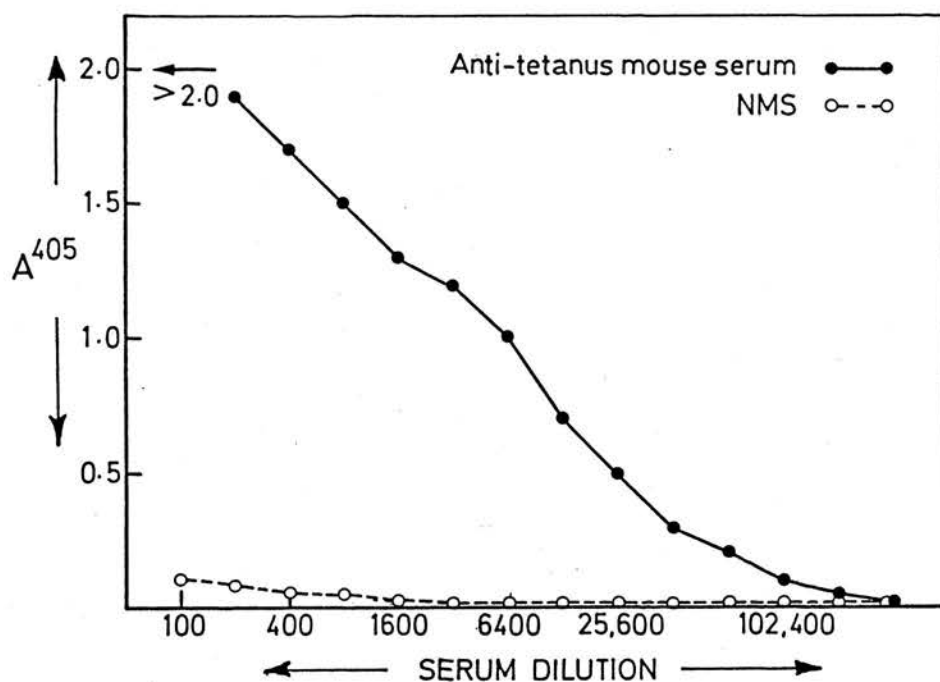
TABLE C.1.4-1

OPTIMAL DILUTIONS FOR Ig-ISOTYPES ANTIBODY

Anti-mouse Ig-isotype	Optimal dil. for SEI
M	1:70
A	1:100
G1	1:4000
G2a	1:700
G2b	1:3500
G3	1:130

Dilutions of isotype specific antibody (2nd or bridging antibody) required to saturate pooled anti-tetanus mouse serum (primary antibody) at 1:200 dilution (15  $\mu$ g) to give O.D. 405 of 1.0. The above are final dilutions after dividing by 3 to ensure excess of antibody for routine assay (SEI).

FIG. C.1.4-3: DETERMINATION OF NON-SPECIFIC BINDING OF  
SEI ASSAY



Dilutions of both positive and negative sera for tetanus antibody were tested against fixed dilution (1:4000) of antimouse IgG<sub>1</sub>. Results show no non-specific binding by NMS and a high level of IgG<sub>1</sub> antibody in anti-tetanus mouse serum.

FIG. C.1.4-4: SEI RESULTS OF Ig-ISOTYPE LEVELS OF POOLED MOUSE ANTI-TETANUS SERUM

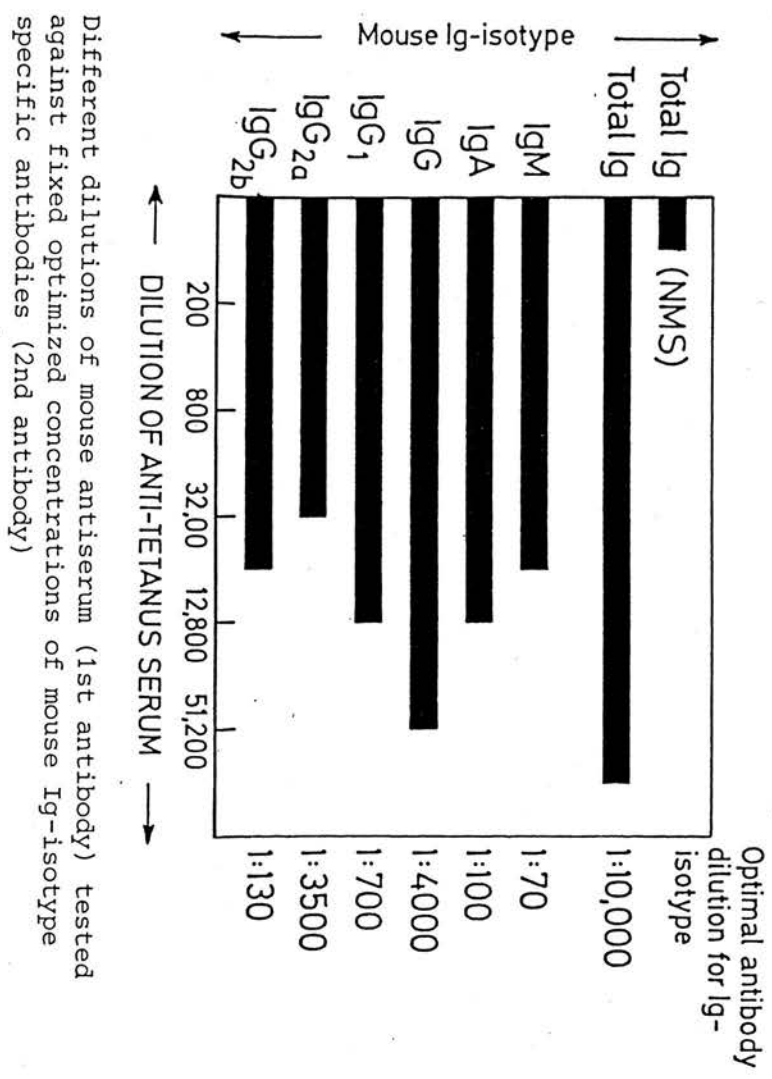
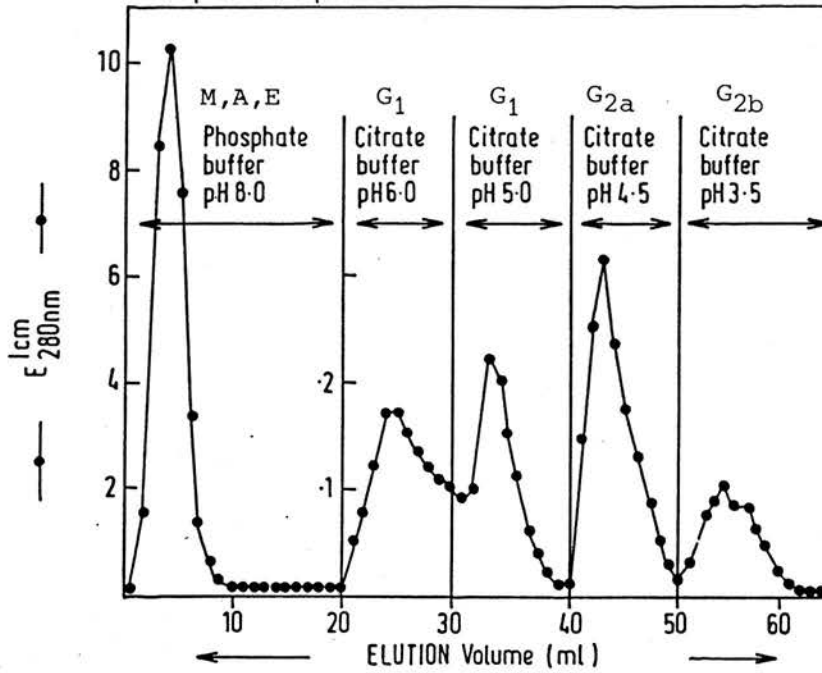


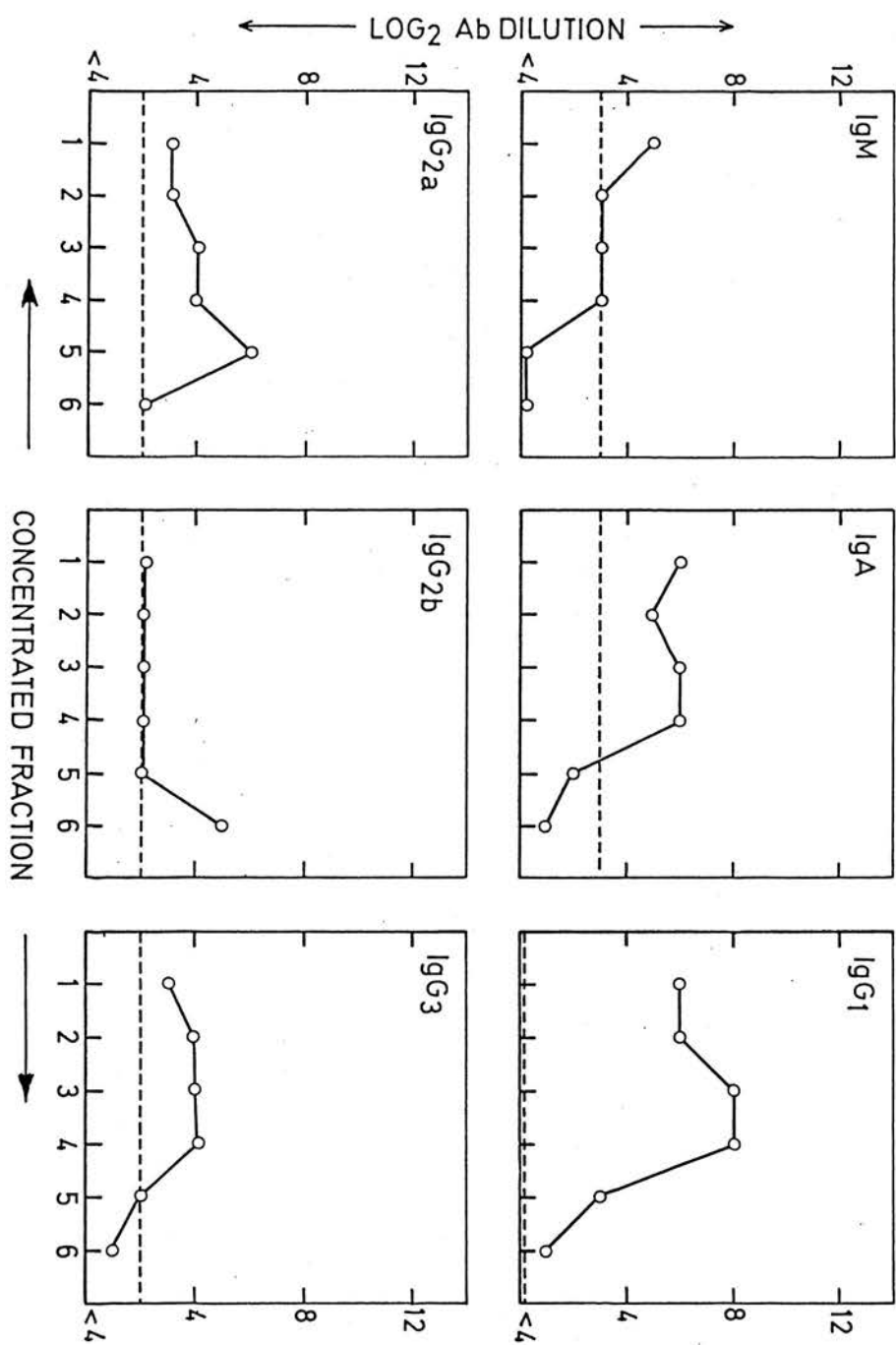
FIG. C.1.4-5

Ig-Isotype separation of antitetanus mouse serum (BALB/c)  
on protein A sepharose



Each of the 10 eluted fractions were pooled and concentrated. The Ig-isotypes of the concentrated fractions were tested by SEI assay (see Fig. C.1.4-6).

FIG. C.1.4-6: SEI RESULTS ON PROTEIN-A SEPHAROSE SEPARATED FRACTIONS OF MOUSE ANTITETANUS SERUM





C.2 CHAPTER 2

ATTEMPTS TO DEVELOP A TECHNIQUE FOR MEASURING THE ANTIGEN BINDING  
CAPACITY AND THE AFFINITY OF ANTI-TETANUS ANTIBODY

## C.2 ATTEMPTS TO DEVELOP A TECHNIQUE FOR MEASURING THE ANITGEN BINDING CAPACITY AND THE AFFINITY OF ANTI-TETANUS ANTIBODY

### 2.1 Introduction

Most antibody tests do not measure the initial interaction of antigen and antibody but secondary phenomena such as agglutination, complement fixation, etc. The ammonium sulphate test, first introduced by Farr (1958) fulfills the need for a primary binding test, and also enables determination of antibody affinity. It measures the capacity of antisera to combine with soluble macromolecular antigens and detects both precipitating and non-precipitating antibody. The Farr technique relies on the fact that some antigens are not precipitated by concentrations of ammonium or sodium sulphate that precipitate immunoglobulins. If such antigens are radioactively labelled and added to an antiserum, any antigen combining with specific antibody will be precipitated with half saturated ammonium sulphate while unbound antigen will remain in the supernatants. The amount of radioactivity precipitated is an indication of the amount of antibody present. In addition because this procedure allows simultaneous determination of the amount of bound and free antigen, the affinity constant of the antibody concentration can be measured by employing the Scatchard equation (Scatchard, 1949):-

$$\frac{B}{F} = (AB_t - B) K_a$$

where B and F are the concentrations of the bound and free antigen respectively and  $AB_t$  is the molar concentration of combining sites on the antibody molecules at the dilution of antiserum employed. The Scatchard plot is a graph of B/F against B, giving a straight line from

which  $K_a$  can be determined by the slope, and  $Ab_t$  by the intercept on the X axis.

A modified ammonium sulphate precipitation technique as described by Alausa (1975) was used in the present studies with the aim of:-

- (a) measuring the binding capacity and binding affinity of anti-tetanus antibody in unfractionated sera from immunized donors, and hence to select sera with the highest antibody affinity for isolation of specific antibody against tetanus-toxoid by affinity chromatography.
- (b) determining whether the antibody of high affinity is lost as a consequence of this immunoaffinity purification technique.
- (c) ascertaining the binding affinity of anti-tetanus antibody during the course of immunization.

## 2.2 Materials

2.2.1 Tetanus-toxoid (Wellcome, batch A50 688) was labelled with  $^{125}I$  by the chloramine-T method (Section B.3). The free iodine was removed by Sephadex G-100 gel filtration (Alausa, 1975).

2.2.2 Antisera were obtained from the plasma of donors previously immunized with 0.5 ml tetanus vaccine. Anti-tetanus IgG was obtained from the Protein Fractionation Centre (Edinburgh), details of which are recorded in table of antibodies (Section B.11).

2.2.3 Normal human serum (NHS) was obtained from the Blood Transfusion Service (Edinburgh). It was tested by ELISA and HA techniques and found to be negative for anti-tetanus antibodies. This serum was used as a protein carrier for radioiodinated antigen, as well as a negative

serum control during the experiment.

2.2.4 Saturated ammonium sulphate (SAS): This was prepared and stored at 4°C with some crystals in the flasks to prevent the solution from becoming supersaturated. It was filtered before use and diluted 1:2 with PBS pH 7.2 to make a half saturated solution designated 50% SAS.

## 2.3 Methods

### 2.3.1 Measurement of antigen binding capacity (ABC)

This was carried out at 4°C. Serum dilutions greater than 1:10 were made in 1% NHS in PBS. The same diluent was used to dilute the antigen.

A constant amount of antigen (0.106 µg of protein/0.5 ml) was mixed with 0.5 ml of antiserum at different dilutions. The control tubes were set up in duplicate and included (a) an Ag-control which contained 0.5 ml <sup>125</sup>I-labelled tetanus toxoid alone, and (b) NHS-control which contained 0.5 ml of 1% NHS and 0.5 ml labelled antigen. All the tubes were mixed and incubated overnight at 4°C.

One ml SAS was added to all the tubes except the Ag-controls, the tubes were mixed immediately and then incubated at 4°C for 30 min followed by centrifugation at 2000 rpm for 30 min at 4°C. The supernatants were discarded and the precipitates washed by adding 3.0 ml of SAS/2. This was mixed well and centrifuged as above. The supernatants were discarded and the precipitates were counted in a gamma counter.

Calculations were performed as described by Minden and Farr (1978) and Alausa (1975). Steps involved were as follows:-

(a) % non-antibody bound Ag (%S) =

$$\frac{\text{c.p.m. of Ag control} - \text{c.p.m. of experimental}}{\text{c.p.m. of Ag control} - \text{c.p.m. of NHS control}} \times 100$$

(b) % P (% of Ag specifically bound to Ab) = 100 - %S.

(c) A graph of % P (linear axis) against the reciprocal of the antiserum dilution (log scale) was plotted. From the plot, the antiserum dilution which bound 33% of the antigen was determined (ABC-33). The ABC-33 value for each antiserum was expressed as the  $\mu\text{g}$  labelled antigen bound per ml of undiluted serum. This was obtained by multiplying the dilution of antiserum required to precipitate 33% of the antigen by 0.33 times 0.212 ( $\mu\text{g}/\text{ml}$  of antigen used in the assay).

#### 2.3.2 Determination of the affinity constant ( $k_a$ ) of antitetanus-Ab

Equal volumes of an antiserum dilution which was found to bind 50% of the labelled antigen (ABC-50) was incubated with 7-8 different concentration of labelled antigen in duplicate and a Farr assay performed as before. The controls were (a) Ag-control tubes containing 0.5 ml of each concentration of labelled antigen only, and (b) NHS-control tubes containing 0.5 ml of 1% NHS and 0.5 ml of labelled antigen at each concentration. The radioactivity in the precipitates of each experimental tube (after correction for non-specific precipitation using values obtained from NHS) represents bound antigen (B). The difference between the total activity in the untreated antigen control tubes for each concentration and the bound activity represents the free antigen (F). A graph of (B/F) against (B) was plotted after converting the concentration of bound labelled antigen to moles/litre. In order to do this a molecular weight for tetanus toxoid of 67000 was used (Alausa 1975). Although there was some debate about the molecular weight of tetanus toxoid we decided for comparison sake to use the molecular weight previously employed by Alausa (1975) in determining affinity constant. The affinity constant ( $K_a$ ) for each

antiserum or tetanus IgG was determined from the gradient and is expressed in litres/mole.

## 2.4 Results and Discussion

Preliminary experiments of Ag-binding capacity (Table 1) showed that the assay worked. However later experiments indicated that the assay was not very reproducible. Non-specific binding (high background) appeared to be the main problem. Experiments were therefore undertaken to investigate a number of factors that could have been responsible for the variability.

### 2.4.1 The iodine-labelling of tetanus-toxoid

The labelling procedure may have altered the antigen. In Table 1 and Table 3, two different labelled tetanus-toxoid (Wellcome) preparations were used. Table 1 shows less non-specific binding of antigen by NHS than Table 3, with ABC-33 end point of 1:41 (Fig.1/Table 1) and the ABC of the undiluted antiserum was only 2.7 ( $\mu\text{g/ml}$ ). These values were much higher using the second preparation (Table 3/Fig.3) and the same anti-tetanus IgG. The ABC-33 was 1:360, and 25.2  $\mu\text{g}$  of labelled antigen was bound per ml of undiluted antiserum.

The determination of affinity constant of anti-tetanus IgG is illustrated in Table 2/Fig.2, but the results were not satisfactory. Variability of B/F were observed in the lower range of the antigen concentrations due to antibody being in excess (Table 2). Obviously affinity constant can not be accurately calculated by plotting only two points in the graph. This problem might have been overcome by increasing the amount of antigen to antibody ratio, i.e. selecting ABC-40 values, which determines the appropriate concentration of

antibody to be used in the assay, instead of ABC-60 which was selected in this experiment. This was obtained from Fig.1.

#### 2.4.2 The precipitation procedure

2.4.2.1 Different batches of Amm. Sulphate (SAS) gave different results with respect to the amount of antigen precipitated. The non-specific binding NHS (1:10 dilution) using SAS batch A (No. 07081, M & B Lab. Chemicals, Manchester, U.K.) was only 20% as compared to 60% (Tables 4 and 5) using SAS batch B (No. 48870). Batch A (Table 4) also showed that 91% of the antigen was bound to anti-tetanus IgG, which is the highest percentage achieved in this trial.

There are certain factors that must be considered in preparation of SAS (Minden & Farr, 1978). Its specific gravity must be between 1.2332-1.248. This was not measured in the above batches; therefore, batch A was either a purer Amm. Sulphate, or happened to have the required specific gravity. It has also been suggested (Minden and Farr, 1978) that borate buffer should be used instead of PBS to make the 50% SAS solution. This was tested in Table 6 (Borate buffer) as compared to Table 7 (P.B.S. 7.2). It shows that the percentage of bound-Ag is, if anything, slightly lower using borate buffer, although no conclusion can be drawn as they were not directly compared in the same assay.

#### 2.4.2.2 Comparison of Amm. Sulphate with Sodium Sulphate

Table 7 shows that the tetanus-toxoid is not completely soluble in 50% Amm. Sulphate, therefore 16% Sodium Sulphate was used in parallel with 50% Amm. Sulphate in attempts to decrease the non-specific

background. As Table 7 shows, the 16% Sodium Sulphate had the effect of reducing the amount of radioactivity (c.p.m.) non-specifically bound. On the other hand Sodium Sulphate gave lower specific binding values with higher antiserum dilutions. In neither case was 100% of antigen bound.

The reagents were also compared for their ability to precipitate antibody using "<sup>125</sup>I-labelled anti-tetanus IgG". 87% was precipitated by Amm. Sulphate and 90% by Sodium Sulphate (see Table 7).

#### 2.4.2.3 NHS Control

The possibility existed that some of the "non-specific" binding of antigen could be due to antibody in the NHS control, even though it was negative in anti-tetanus antibody activity by haemagglutination assay. In an attempt to reduce the non-specific binding by NHS which is used as sample diluent as well as antibody-negative control, the NHS was absorbed by affinity chromatography on a tetanus toxoid coupled CNBr-activated sepharose-4B column (as described in Section B.6 & C.5). the PBS eluates which were also negative for anti-tetanus activity by the haemagglutination assay were used as a NHS control in the assay in comparison with non-absorbed NHS (Table 7). There was no difference between the absorbed and non-absorbed control sera.

#### 2.4.3 Heterogeneity of tetanus-toxoid

One of the factors that may have been responsible for the variability of the assay was thought to be the heterogeneity of the Wellcome tetanus toxoid. A series of studies were carried out to investigate the physiochemical characteristics of different tetanus toxoid preparations, details of which are described in Section C.3.



The results clearly indicated the heterogeneity of Wellcome tetanus toxoid; it consisted of three main protein peaks by G-200 separation, from which only 4.5S peak contained the toxoid activity. When fractions were tested for antigen solubility, they were all 50% soluble in 50% SAS. However this solubility was also observed with the pure and homogeneous Connaught tetanus-toxoid, which indicates that the non-specific binding observed in the antibody affinity assay was probably not due to the heterogeneity of the Wellcome tetanus-toxoid. Nevertheless, the pure tetanus toxoid labelled more efficiently with  $^{125}\text{I}$ , giving a TCA precipitability of around 90% (results in Section C.3) as compared to that of Wellcome toxoid ( $71\% \pm 4.5$ ). Therefore the assay could have been repeated using the pure tetanus-toxoid (not available at the time), or labelling ( $^{125}\text{I}$ ) the 4.5S fraction of the Wellcome tetanus-toxoid which showed the highest antigenic activity. However as discovered later (Tanavoli et al. 1978) others have also observed that 50% SAS has resulted in excessive non-specific precipitation in the Ag-Ab system used. As a result they had to use a mixture of ethanol-ammonium acetate as the precipitating reagent in their modified Farr assay.

In view of the difficulties experienced with the Farr assay, further studies in this direction were abandoned.

## 2.5 Conclusions

The assay has proved to be irreproducible as discussed. It is conceivable that the assay could be improved by taking into consideration the following points, though this remains to be tested.

2.5.1 There are reasons to believe that the chloramine-T or the sodium

metabisulphite used to reduce the excess chloramine-T may damage the protein. The modified labelling technique described in Section B.3 might have proved more suitable.

2.5.2 It is possible that some of the solubility problems might have been minimised by using a highly purified tetanus toxoid (Connaught) or the 4.5S fraction of Wellcome toxoid.

2.5.3 Alternatively the non-specific precipitation of toxoid might have been overcome by using in the assay the 50% SAS soluble fraction of tetanus toxoid.

2.5.4 Cleaner separations of free and bound antigen might have been achieved using a mixture of ethanol-ammonium acetate as the precipitating reagent rather than 50% Ammonium Sulphate.

2.5.5 Excess antibody undoubtedly contributed to the variability in Ab-affinity measurement ( $K_a$ ). Therefore either higher concentrations of the antigen, or higher dilution of the antiserum (less than ABC-60) should have been used.

TABLE C.2-1

## DETERMINATION OF AG-BINDING CAPACITY OF HUMAN ANTITETANUS IgG

ABC-33 = 2.7  $\mu$ g/ml (see Fig. 1)

Sample	Dilution	C.p.m.	% Bound-Ag*
Ag-Control	0.212 $\mu$ g/ml	4613	-
NHS	1/100	3	-
Antitetanus IgG	1/10	2622	100
"	1/50	598	23
"	1/100	512	19
"	1/200	484	17
"	1/400	437	16
"	1/800	412	16
"	1/1600	391	14

Preliminary experiment which showed that this assay is reproducible and specific. No non-specific binding was observed with NHS.

\* % Bound-Ag, considering the TCA precipitability of the labelled antigen calculated by:  $\frac{\text{Sample c.p.m.} - \text{NHS c.p.m.}}{\text{TCA precipitate c.p.m.}} \times 100$

FIG. C.2-1

From the plot, the tetanus-antiserum dilution which bound 33% of the tetanus toxoid (ABC-33) =  $2.7 \mu\text{g}$  of labelled Ag per ml of undiluted serum which was obtained by multiplying the dilution of antiserum (1:41) required to precipitate 33% of the antigen by 0.33 times  $0.212 \mu\text{g/ml}$  of the antigen used in the assay).

FIG. C.2-1

Ag-BINDING CAPACITY (ABC) OF ANTI-TETANUS IgG

ABC-33 = 2.7  $\mu$ g of Ag/ml of U.D. serum

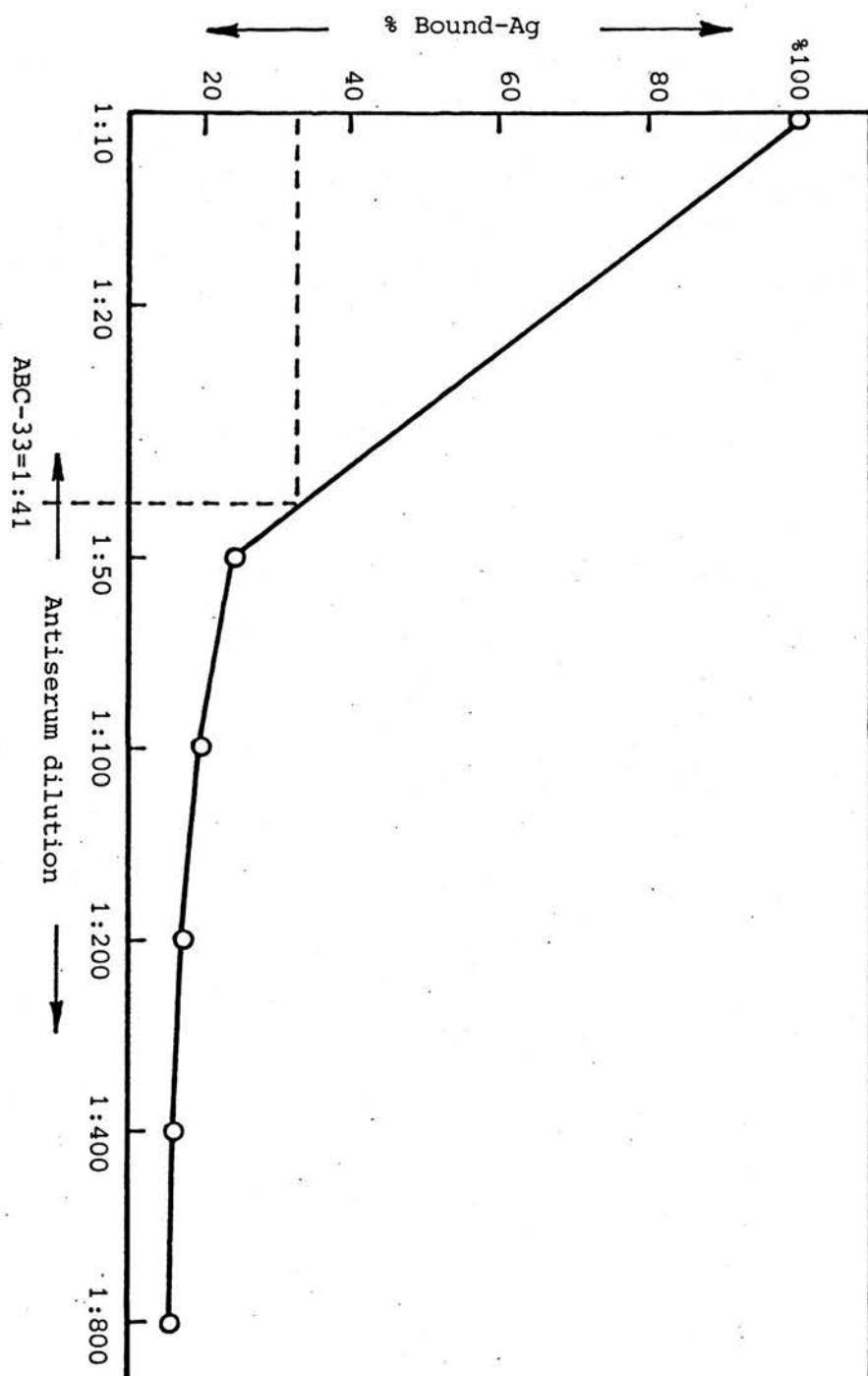


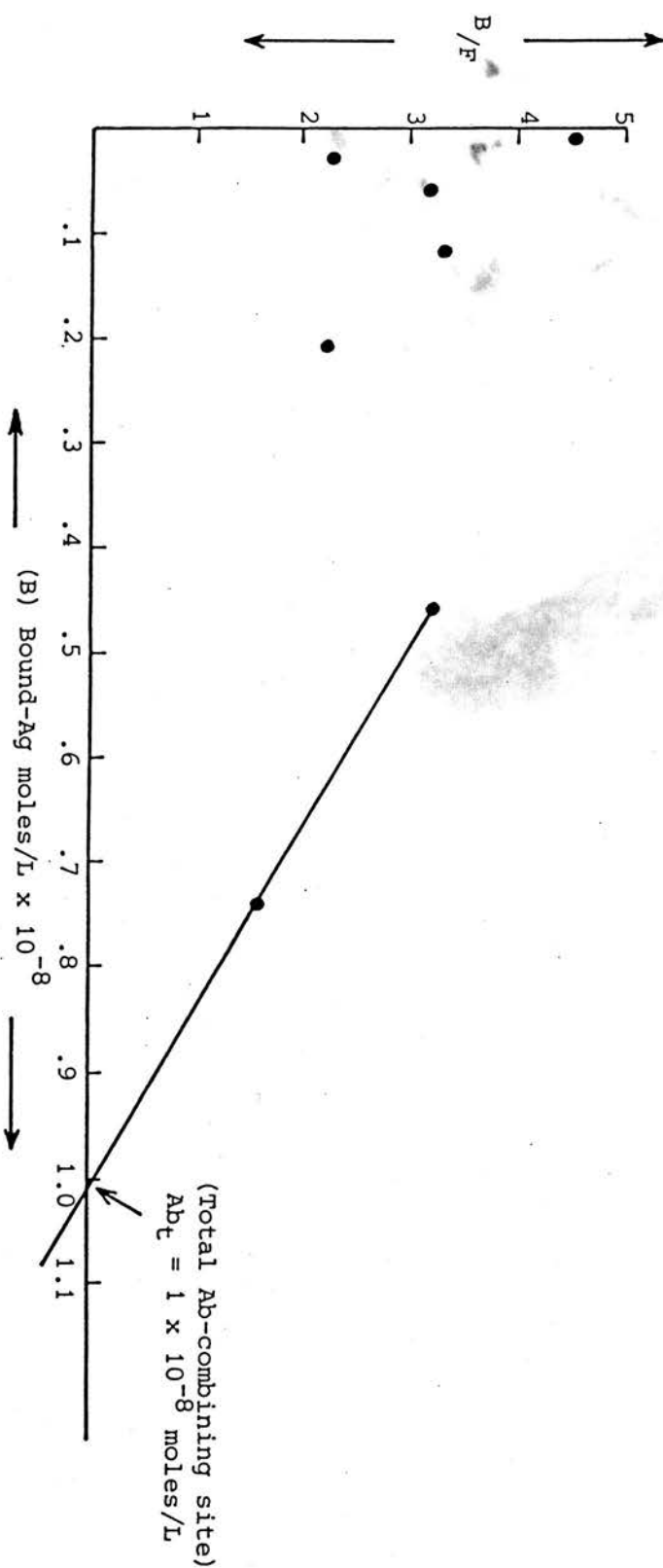
TABLE C.2-2

DETERMINATION OF AFFINITY CONSTANT OF ANTITETANUS IgG, TAKING ABC-60 VALUES OBTAINED FROM FIG. 1 USING THE SAME LABELLED ANTIGEN. A GRAPH OF B/F AGAINST CONC. OF BOUND Ag (moles/l) IS PLOTTED (SEE FIG. 2)

Ag-Conc. $\mu\text{C/ml}$	Ag-Control C.p.m.	NHS Control C.p.m.	Exp. Antitetanus IgG C.p.m.	Bound-Ag C.p.m. (B)	Free-Ag(F) C.p.m.	B/F	Conc. of Bound-Ag Moles/l
0.01	260	31	244	213	47	4.5	$0.012 \times 10$
0.025	654	76	530	454	200	2.3	$0.03 \times 10$
0.05	1322	179	1187	1008	314	3.2	$0.06 \times 10$
0.1	2717	363	2448	2085	632	3.3	$0.12 \times 10$
0.2	4830	687	4021	3334	1496	2.2	$0.21 \times 10$
0.4	10078	1610	9301	7691	2387	3.2	$0.46 \times 10$
0.8	20795	3835	16590	12755	8040	1.6	$0.74 \times 10$

FIG. C.2-2

SCATCHARD PLOT FOR MEASUREMENT OF AFFINITY CONSTANT OF ANTI-TETANUS IgG  
 $K_a = 5.9 \times 10^8$  litre/mole using Scatchard equation  $[B/F = (Ab_t - B) K_a]$



Bound (B) and free (F) antigen values listed in Table 1 were plotted. The affinity constant ( $K_a$ ) for antiserum is determined from the gradient and is expressed in litre/mole.

TABLE C.2-3

DETERMINATION OF AG-BINDING CAPACITY OF HUMAN ANTITETANUS USING

SECOND BATCH OF LABELLED TETANUS-TOXOID. ABC-33 = 25.2  $\mu\text{g/ml}$ 

Sample	Dilution	C.p.m.	% Bound-Ag
Antitetanus IgG	1:10	13411	93
	1:20	13194	88
	1:40	12832	79
	1:80	12413	68
	1:160	11816	54
Antitetanus Serum	1:10	12563	72
	1:20	12313	67
	1:40	11755	53
	1:80	10738	26
	1:160	11078	36
NHS	1:100	9655	-
AG-Control	0.212 $\mu\text{g/ml}$	15230	-

Same experiment as shown in Table 1, except a different batch of Wellcome vaccine was used. Note that this antigen's binding capacity was much higher (10x). 25.2  $\mu\text{g}$  of labelled antigen was bound per ml of tetanus-IgG (see Fig.3).



FIG. C.2-3

Ag-BINDING CAPACITY OF ANTI-TETANUS IgG  
 ABC-33 = 25.2µg of Ag/ml of U.D. IgG

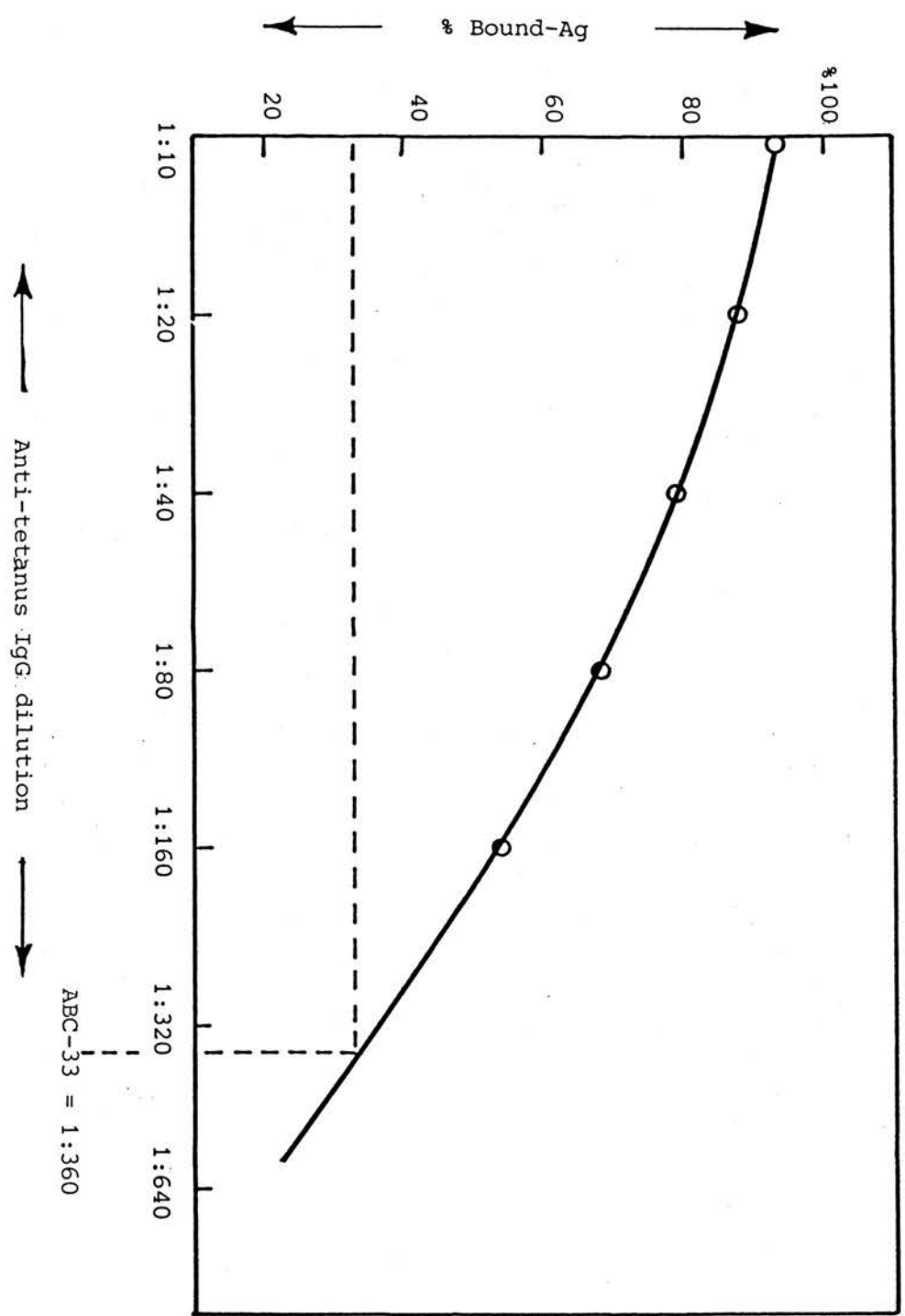


TABLE C.2-4

DETERMINATION OF Ag-BINDING CAPACITY USING SAS BATCH A

Sample	Dilution	C.p.m.	% Bound-Ag
NHS	1:10	4471	20
	1:100	1191	-
Antitetanus Serum	1:10	11368	61
	1:100	5017	23
Antitetanus IgG	1:10	16192	91
	1:100	9098	48
	1:200	7783	40
Ag-Control	0.212 $\mu$ g/ml	17749	

This batch of SAS shows (a) lower non-specific binding of Ag by NHS, and (b) higher percentage of bound Ag (to IgG) as compared to Table 5.

TABLE C.2-5

DETERMINATION OF Ag-BINDING CAPACITY USING SAS BATCH B

Sample	Dilution	C.p.m.	% Bound-Ag
NHS	1:10	12780	60
	1:100	5379	-
Antitetanus Serum	1:10	14082	70
	1:100	8171	23
Antitetanus IgG	1:10	15678	83
	1:100	15346	81
	1:200	7469	17
Ag-Control	0.212 $\mu\text{g/ml}$	17749	

This batch of SAS shows (a) higher non-specific binding of Ag by NHS, and (b) lower percentage of bound-Ag (to IgG) as compared to Table 4.

TABLE C.2-6

DETERMINATION OF Ag-BINDING CAPACITY USING FRESH BATCH OF SAS,  
AND BORATE BUFFER INSTEAD OF PBS-7.2 AS DILUTING FLUID

Sample	Dilution	C.p.m.	% Bound-Ag
Antitetanus Serum	1:10	5024	32
	1:20	5083	32
	1:50	5030	30
	1:100	4660	21
	1:200	4724	22
	1:400	4513	17
NHS	1:100	3841	-
Ag-Control	0.212 $\mu\text{g/ml}$	7760	-

The percentage of bound-Ag is slightly lower using borate buffer than using P.B.S. (as in Table 7). It seems however that the nature of the diluting fluid has no dramatic effect on the test results.

TABLE C.2-7

COMPARISON OF AMM. SULPHATE V. SODIUM SULPHATE PRECIPITATION  
FOR DETERMINATION OF AG-BINDING CAPACITY (ABC)

Sample	Dilution	C.p.m.	% Bound-Ag	Ppt. Solution
Antitetanus Serum	1:10	5384	40	50% $(\text{NH}_4)_2\text{SO}_4$
	1:20	5057	34	
	1:50	4867	30	
	1:100	4640	25	
	1:200	4031	12	
	1:400	3938	10	
NHS Non-absorbed	1:100	3466	-	
NHS absorbed	1:100	4020	-	
$^{125}\text{I}$ -labelled tetanus IgG	1:20	618559	87	
Antitetanus Serum	1:10	4197	40	16% $\text{Na}_2\text{SO}_4$
	1:20	3742	33	
	1:50	2516	14	
	1:100	2196	10	
	1:200	2025	7	
	1:400	1586	1	
NHS Non-absorbed	1:100	1583	-	
NHS absorbed	1:100	1534	-	
Ag-Control	0.212 ug/ml	8261	-	
$^{125}\text{I}$ -labelled tetanus IgG (alone)	1:20	636725	90	

In this comparison study the non-specific binding of the NHS and of more diluted samples were decreased using sodium sulphate as precipitating agent. The results of absorbed-NHS did not differ from the non-absorbed.

C.3 CHAPTER 3

STUDIES ON TETANUS TOXOID PREPARATIONS USED IN THIS PROJECT

### C.3 STUDIES ON TETANUS TOXOID PREPARATIONS USED IN THIS PROJECT

#### 3.1 Introduction

Tetanus toxin is a powerful neurotoxin produced by Clostridium tetani, an anaerobic organism, which is widely dispersed in nature, and enters the body through the wound. Although the toxin has been under investigation for many years, surprisingly little is known about its mode of action at the molecular level or about its chemical structure and nature.

There has been disagreement in the past about the molecular weight of the purified toxin. It is currently believed that the tetanus toxin contains no lipid or carbohydrate moieties and is a simple protein of approximately 150,000 daltons. It exists in two distinct forms: "intracellular" (extract or unnicked) toxin which is a single chain, 150,000-dalton polypeptide, and "extracellular" (filtrate or nicked) toxin. This is a protease-cleaved form composed of two disulphide-linked polypeptide chains. These are referred to as the " $\alpha$ ", "light" or "L"-chain (50,000 daltons) and the " $\beta$ ", "heavy" or "H"-chain (100,000 daltons) (Di Mari et al., 1982, Craven et al., 1973). It turns out that the two chains must be tightly associated together by non-covalent forces in the intact molecule, because even under conditions when the covalent disulphide bonds are broken, it is not easy to separate the two chains quantitatively. Several workers have purified them by gel-permeation chromatography in the presence of denaturing agents (Matsuda and Yoneda, 1975), but in all cases the purification is incomplete: the H-chain which comes through the column first is contaminated with L-chain, although the L-chain is comparatively pure. The individual chains have little or no toxicity;

what toxicity is found can be reasonably ascribed to contamination with the other chain (van Heyningen, 1980).

The chief interest in the protein chemistry of tetanus toxin from a practical point of view has been in the mechanism of production of toxoid, which is defined as a protein so treated that it can elicit the production of antibodies that neutralise the activity of toxin, but is not itself toxic. Toxoiding is usually done by prolonged incubation of the protein in formaldehyde solution (Loewenstein, 1909). It is well documented that formaldehyde is a cross-linking reagent, forming intra and interchain methylene cross linkage (Murphy, 1967; Bezzini et al., 1974). The cross-linking reactions could explain the different values reported for the size of the tetanus toxoid molecule (Largier et al., 1956; Raynaud et al., 1960 and Latham et al., 1965).

Of all the infectious diseases, immunization against tetanus has proved one of the most effective (Haberman, 1978). The great success of prophylaxis against the disease achieved by mass immunization with tetanus toxoid, has meant that the preparation is produced commercially in large amounts and variety all over the world. The tetanus toxoid preparation used in most of our studies was a tetanus vaccine in simple solution (Wellcome). This antigen was regularly used for hyperimmunization of human volunteers for the production of tetanus immunoglobulin used in passive protection against tetanus.

As a valuable adjunct to our proposed affinity chromatography studies we decided to investigate the heterogeneity of various commercially prepared tetanus toxoid preparations. The products chosen were (a) different batches of tetanus vaccine (Well<sub>1</sub>-TT), (b) a more potent form of tetanus toxoid from the same manufacturer (Well<sub>2</sub>-TT), and (c) a highly purified tetanus toxoid produced by Connaught



Laboratories (Conn-TT). It should be stressed that the latter two preparations were not licensed for human use.

The tetanus toxoids were investigated for their purity, distribution and physico-chemical characteristics using the following methods of analysis:-

- (a) Gel filtration (trace labelled) using Sephadex G-200.
- (b) Haemagglutination Inhibition Assay.
- (c) Ammonium Sulphate and TCA precipitation.

### 3.2 Materials and Methods

#### 3.2.1 Iodination of tetanus toxoids

All batches of Well<sub>1</sub>-TT (A52111, A52109, A53255) except batch A50688 were labelled with <sup>125</sup>I by the Chloramine-T method using cysteine-HCl as a reducing agent. The iodination procedure was as described in Section B.3. This procedure was also used to iodine-label Well<sub>2</sub>-TT and Conn-TT. The iodinated fractions were stored in 200  $\mu$ l aliquots at -20°C.

#### 3.2.2 G-200 Sephadex gel filtration

Sephadex G-200 (which separates protein in the MW range between 5000-250,000) was used. All the experiments were performed at 4°C. The gel was swollen and the column was packed as described in Section B.4. The column (containing 0.05% NaN<sub>3</sub>) was stored at 4°C, and was used repeatedly throughout the study. Before each run the column was equilibrated with at least one column volume of PBS buffer.

The tetanus toxoid sample under test was dialyzed (against PBS overnight at 4°C) and then concentrated to approximately 1.5 mg of

protein per ml. A small amount of the same toxoid which has been labelled was added as a marker. This gave approximately  $10^5$  counts per minute. The sample was loaded into the column and eluted at 3 ml per hour. Fractions (200) of 1.5 ml were collected on an LKB automatic fraction collector. The  $E_{280\text{ nm}}^{1\text{ cm}}$  of the fractions were monitored, and later measured by the Unicam SP-500 spectrophotometer to determine protein concentration. Aliquots (100  $\mu$ l) of each fraction were counted in a gamma counter, and the counts (per 100 sec) and  $E_{280\text{ nm}}^{1\text{ cm}}$  values were plotted. The fractions composing each peak were pooled and concentrated using a Minicon Concentrator. The total protein content (as in Section B.1), radioactivity and antigenic activity (using HIA - Section B. 9) of the concentrated fractions were determined. The TCA precipitable (10% v/v) and ammonium sulphate precipitable (50% saturation) counts were determined.

In one study by the gel filtration, characteristics of two preparations were simultaneously compared by labelling one preparation (Well<sub>1</sub>-TT) with  $^{131}\text{I}$ , and the other (Well<sub>2</sub>-TT) with  $^{125}\text{I}$ . The two samples were then mixed and subsequently fractionated on the G-200 Sephadex column. The radioactivity of both iodines in the fractions were measured in a dual channel system on gamma counter (LKB 1260 Multigamma II, Tarku, Finland), and results were compared.

It should be stressed that before each tetanus toxoid separation run, the G-200 column was calibrated by running normal human serum (NHS 1.0 ml) as a standard. The elution profiles of the various tetanus toxoid samples were compared with that obtained with normal human serum.

### 3.3 Results

#### 3.3.1 A comparison of different batches of Well<sub>1</sub>-TT

Initially different batches of the same tetanus toxoid preparation, namely Well<sub>1</sub>-TT were compared. The G-200 separation profiles obtained are shown in Fig.1, Fig.2 and Fig.3. All the batches exhibited similar heterogeneity consisting of two main protein peaks (19S, 4.5S) and a L.M.W. peak. When fractions of each peak were pooled and concentrated only the 4.5S peak contained antigenic activity demonstrable by the HIA test (Fig.4).

The radioiodine labelling capacity of this tetanus toxoid (Well<sub>1</sub>-TT) appeared reproducible from batch to batch,  $68\% \pm 6.7$  being TCA precipitable. However this was lower than observed with the other toxoid preparations. Total protein concentrations were also similar in different batches (See Table 1).

#### 3.3.2 A comparison of the two different tetanus toxoid preparations from same source

A different preparation of tetanus toxoid (PX433) designated here as Well<sub>2</sub>-TT was compared to Well<sub>1</sub>-TT preparation. Well<sub>2</sub>-TT gave higher binding to  $^{125}\text{I}$  in protein iodination (as indicated by TCA precipitation), and higher tetanus toxoid activity was observed (see Table 1).

On G-200 separation the elution profile observed (Fig.5) was similar to that noted with the Well<sub>1</sub>-TT (Figs. 1-3). In the study in which the two tetanus toxoids were trace labelled with different isotopes of iodine, similar elution profiles were obtained with both preparations (Fig.6).

### 3.3.3 A comparison of tetanus toxoid from two different sources

A purified and concentrated tetanus toxoid (Conn-TT) was compared to Well-TT. The results are summarised in Table 1. The Conn-TT was more concentrated (in mg of protein per ml), with greater tetanus toxoid activity (HIA-titre), and appeared to label better than either of the Wellcome tetanus toxoids. Furthermore on the basis of gel filtration studies (see Figs. 7 & 8) it was obvious that the Conn-TT is a purified product. It consisted (unlike Well-TT) of only one main protein peak (4.5S) containing all of the tetanus toxoid antigenicity (Fig. 4).

It should be noted however that with all preparations much of the radioactivity associated with labelled products could not be precipitated by 50 percent ammonium sulphate.

### 3.4 Discussion

Of the tetanus toxoids in this study, the Conn-TT preparation proved to be the purest and have the highest tetanus toxoid activity.

The Well-TT preparations exhibited identical elution profiles from batch to batch and preparation to preparation. All showed similar degrees of heterogeneity. The peak containing most of the toxoid activity was 4.5S in Well-TT and in Conn-TT. The latter was homogeneous and gave rise to only one protein peak (4.5S). This finding agrees with the findings of Pillemer et al. (1948), and Largier (1956). These investigators (who were the first to investigate the molecular state of tetanus toxin) observed that the biological activity was associated with a monomer of sedimentation coefficient of 4.5S. They also found that the toxin, after being kept in a neutral isotonic solution at 4°C for a few days, was partly converted to an atoxic dimer. The "spontaneous toxoid" thus formed exhibited an apparent sedimentation

coefficient of 7.0S.

More recent investigations have not confirmed the work of Pillemer nor of Largier and their associates. In fact, toxin preparations exhibiting similar toxic characteristics to Pillemer's monomer, were found to have sedimentation coefficients ranged from 6.0 to 7.1S (Dawson et al., 1968; Murphy et al., 1967 and Raynaud et al., 1960). The cross-linking reaction between the toxin molecules or between toxin and other proteins in impure preparations could influence the size, distribution and purity of the toxin. During the toxoiding process, the toxin molecules may undergo polymerization. This increases with increasing concentration of formaldehyde and of protein (Murphy, 1967).

Chemical modification with reagents other than formaldehyde that might be expected to lead to more specific and easily investigated changes should be a more useful approach. In this connection it is interesting to note that tetranitromethane has already been used (Bizzini et al., 1973).

The preparation of pure toxoid may best be achieved by formalin treatment of toxin that has undergone prior purification (Dawson et al., 1968), for this would reduce the chance of complexing with irrelevant contaminants. It appears that the Conn-TT which proved the purest in this study may have been purified prior to toxoiding.

In other experiments, such as protein iodination of the three toxoids, the Conn-TT showed the highest binding to  $^{125}\text{I}$ . This was indicated by TCA precipitation of the labelled proteins. However, much of the radioactivity of all the preparations could not be precipitated by half saturated ammonium sulphate. This is particularly relevant to the Farr assay (used to measure the affinity of tetanus antibody)

discussed earlier (Section C.2).

In the present section the physicochemical properties of different toxoid preparations were compared. The reports of the comparative studies such as coupling efficiency (to CNBr-activated sepharose), immunogenicity, etc. are to be found elsewhere in this thesis (Sections C.5 and C.4). It is apparent from these studies that the Conn-TT is less heterogeneous than either of the Well-TT preparations. Whether or not this pure preparation is more effective at eliciting anti-tetanus antibodies in humans or produces fewer or weaker side reactions on booster immunization remains to be established.

### 3.5 Conclusions

1. Both Wellcome tetanus toxoid (Well-TT) preparations are heterogeneous.
2. The protein peak in G-200 separation containing most of the toxoid activities is 4.5S in all of the three preparations.
3. Conn-TT differed from both types of Well-TT in the following respects:-
  - (a) Conn-TT is more concentrated and has higher tetanus toxoid activity.
  - (b) It is radiolabelled with iodine more efficiently.
  - (c) It is more pure, and it contains only one main peak (4.5S).
  - (d) Its cost is subsequently higher.

TABLE C.3-1

A COMPARISON OF DIFFERENT TETANUS TOXOIDS USED IN THIS STUDY

Property	Well <sub>1</sub> -TT	Well <sub>2</sub> -TT	Conn-TT
Potency (LF Unit/ml)	40	791	2500
HIA-titre (TT activity)	1:128	1:8192	1:16384
Total Protein ( $\mu$ g/ml)	312 $\pm$ 11*	570	4600
<sup>125</sup> I labelling (TCA precipitation)	68% $\pm$ 6.7*	88%	90%
G-200 Separation	2 main peaks (19S-4.5S) + L.M.W. Crude	2 main peaks (19S-4.5S) + L.M.W. Crude	1 main peak (4.5S) + L.M.W. Pure
Antigenically active peak	4.5S	7S	4.5S
Ammonium Sulphate- precipitation (50%)	57%	57%	58%

\* Average of 8 experiments on 4 different batches

FIGS. C.3-1, 2 & 3

Radioactivity (○—○) and absorbance at 280 nm (●—●) of G-200 separated fractions of different batches ( $^{125}$ I-labelled) of Wellcome vaccine (Well<sub>1</sub>-TT) is measured. All the batches exhibited similar heterogeneity, consisting of two main protein peaks (19S, 4.5S) and L.M.W. peaks (see Figs. 1-3).



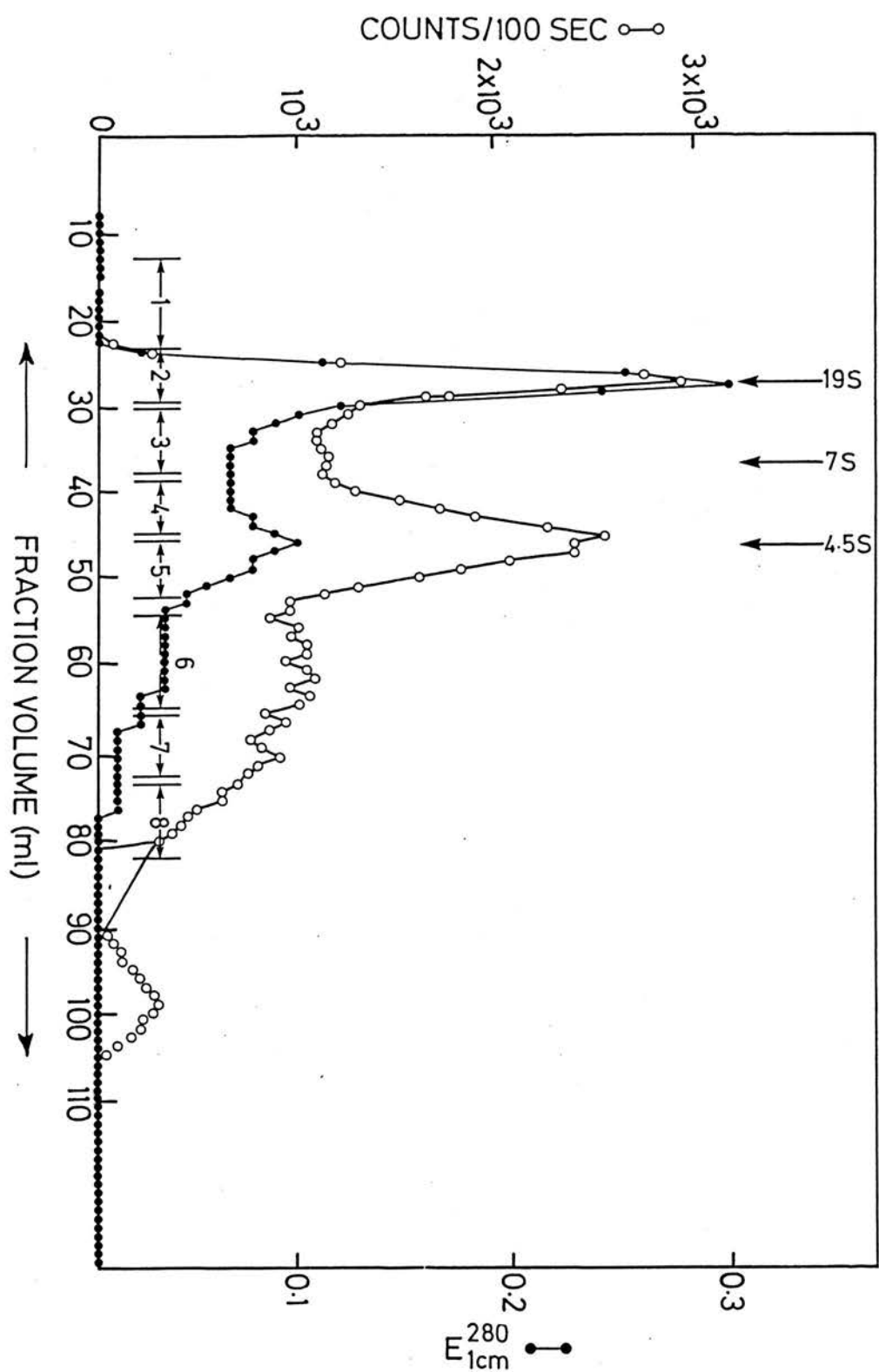


FIG. C.3-1: G-200 SEPARATION OF WELL-TT (BATCH A50688)

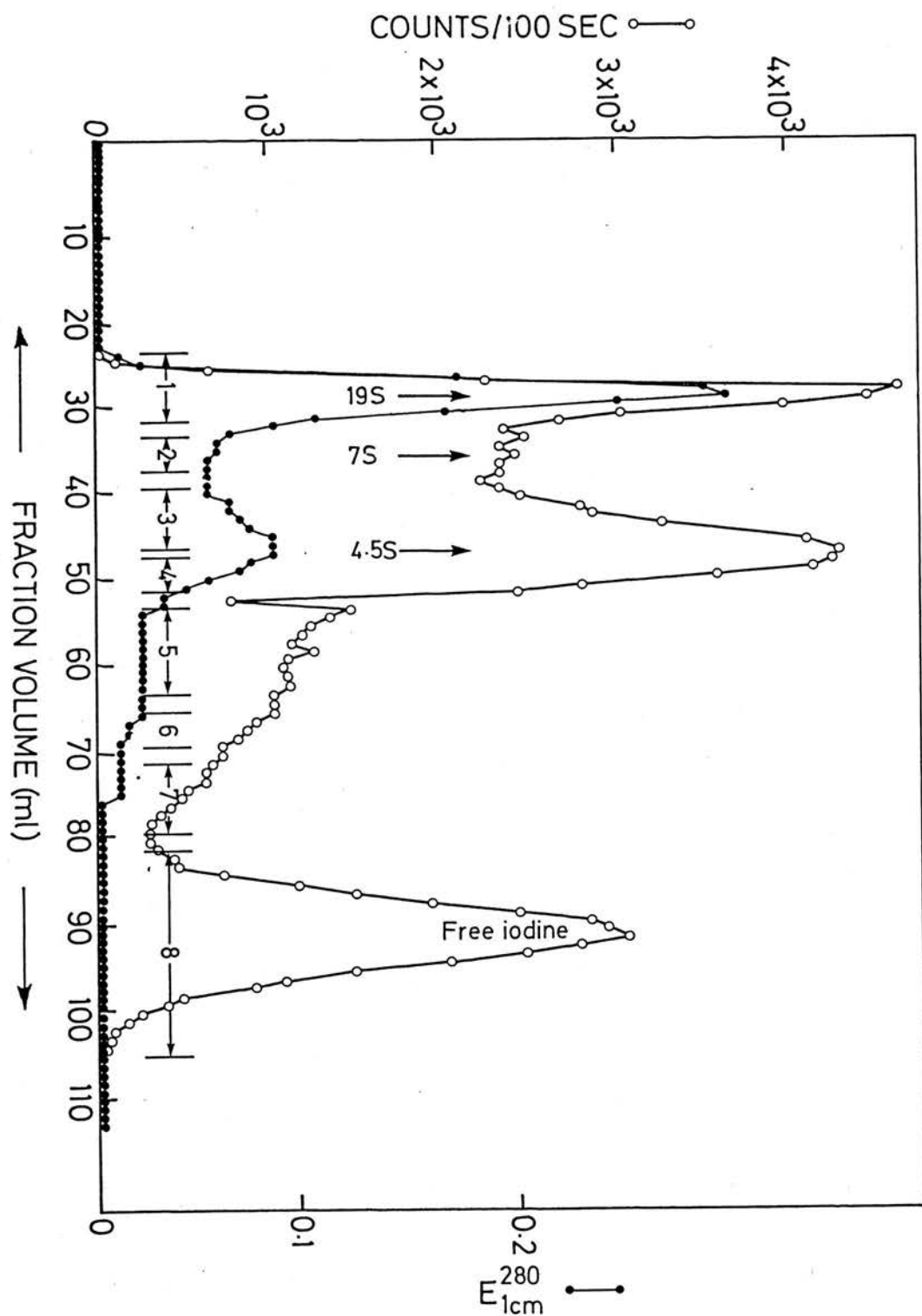


FIG. C.3-2: G-200 SEPARATION OF WELL, -TT (BATCH A52109)

FIG. C.3-3: G-200 SEPARATION OF WEL<sub>1</sub>-TT (BATCH A52111)

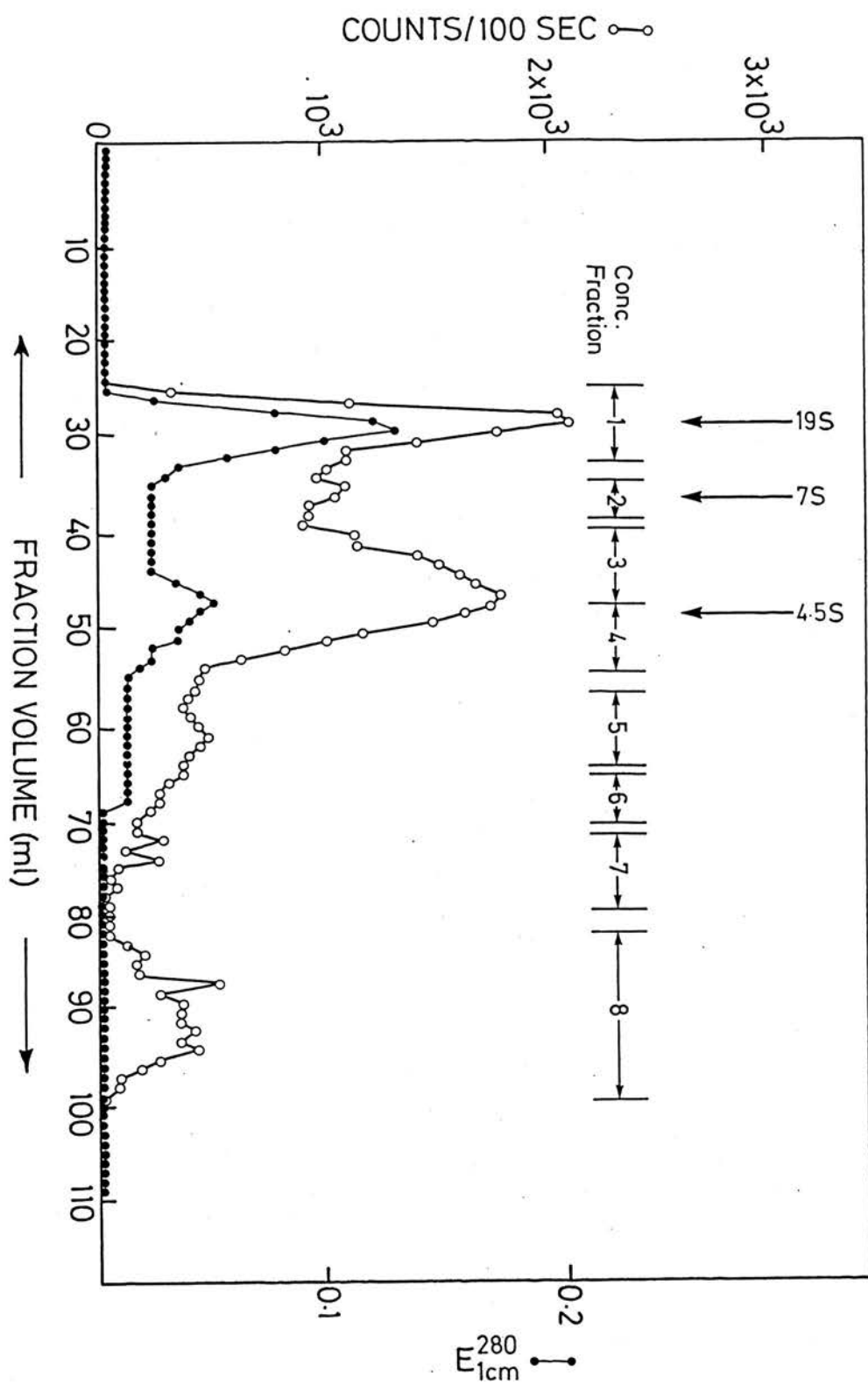
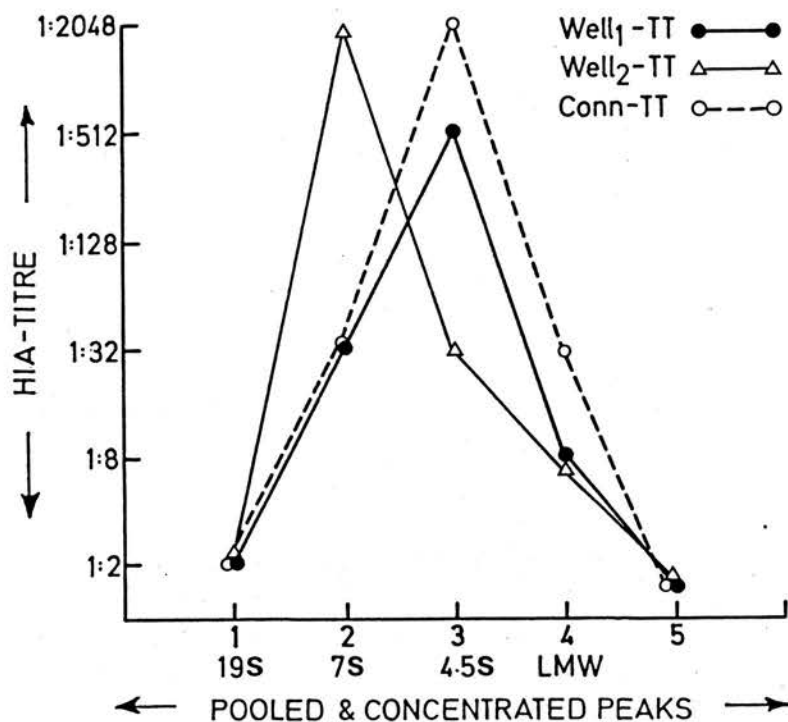


FIG. C.3-4: TETANUS TOXOID ACTIVITIES OF CONCENTRATED FRACTIONS (G-200) OF 3 DIFFERENT PREPARATIONS BY HIA TEST



When tetanus toxoid separated fractions of each peak (Figs. 1,5 & 7) were pooled, concentrated and tested (by Haemagglutination Inhibition Assay), the 4.5S peak contained most of the toxoid activities.

FIG. C.3-5

A different preparation of tetanus toxoid (Well<sub>2</sub>-TT) exhibits very similar elution profile to that of Well<sub>1</sub>-TT, and is as heterogenous (see also Fig.6)

FIG. C.3-5: G-200 SEPARATION OF WELL<sub>2</sub>-TT (PX433)

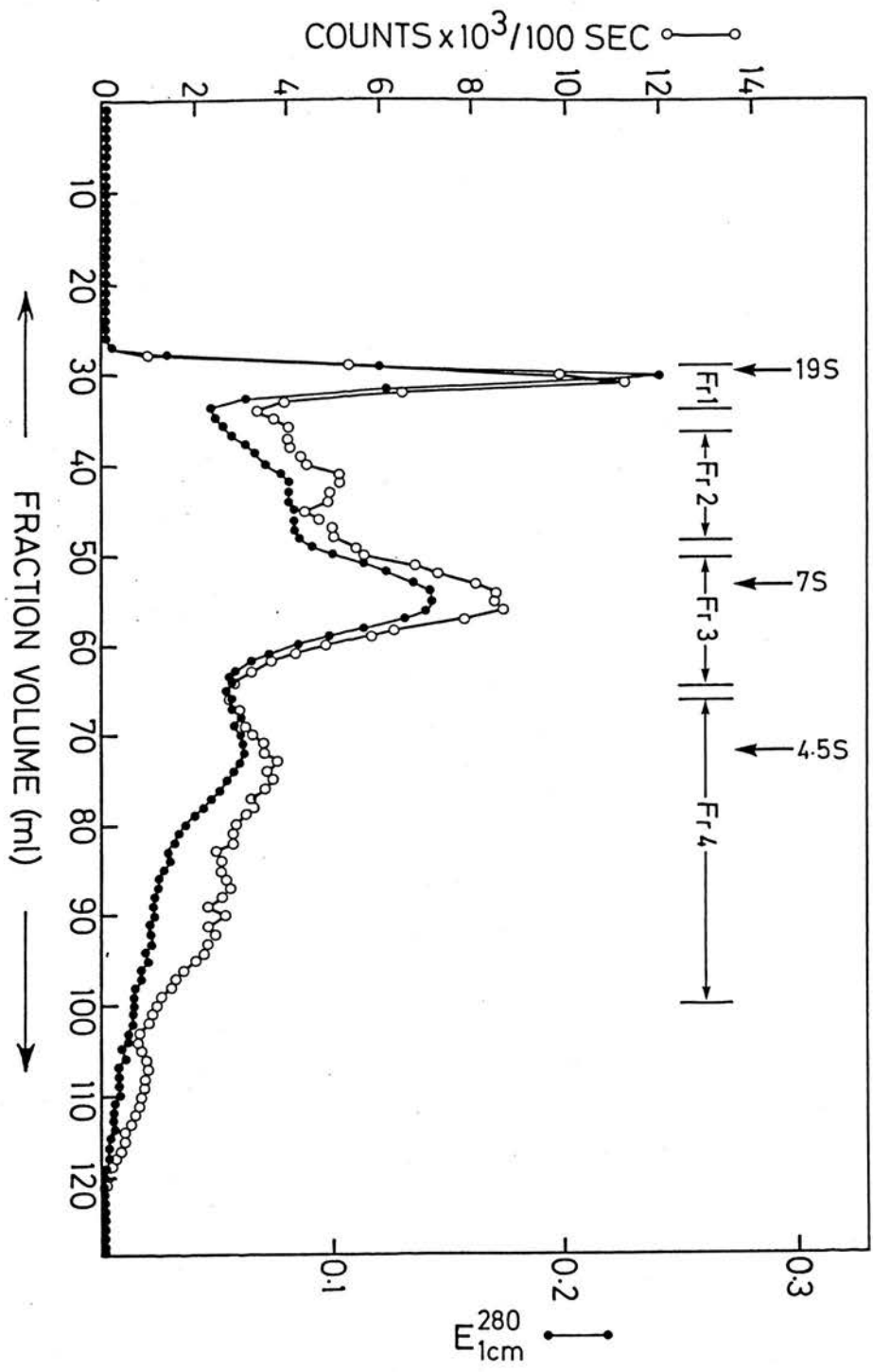


FIG. C.3-6

Comparison of the elution profiles of two different tetanus toxoid preparations (Wellcome). The products were labelled with different isotopes of iodine, (Well<sub>1</sub>-TT ●—●<sup>131</sup>I) and (Well<sub>2</sub>-TT o--o<sup>125</sup>I), and were mixed before elution. They showed similar patterns of heterogeneity, although Well<sub>2</sub>-TT was more concentrated. The radioactivities of the fractions were measured in dual channel system of a gamma counter.

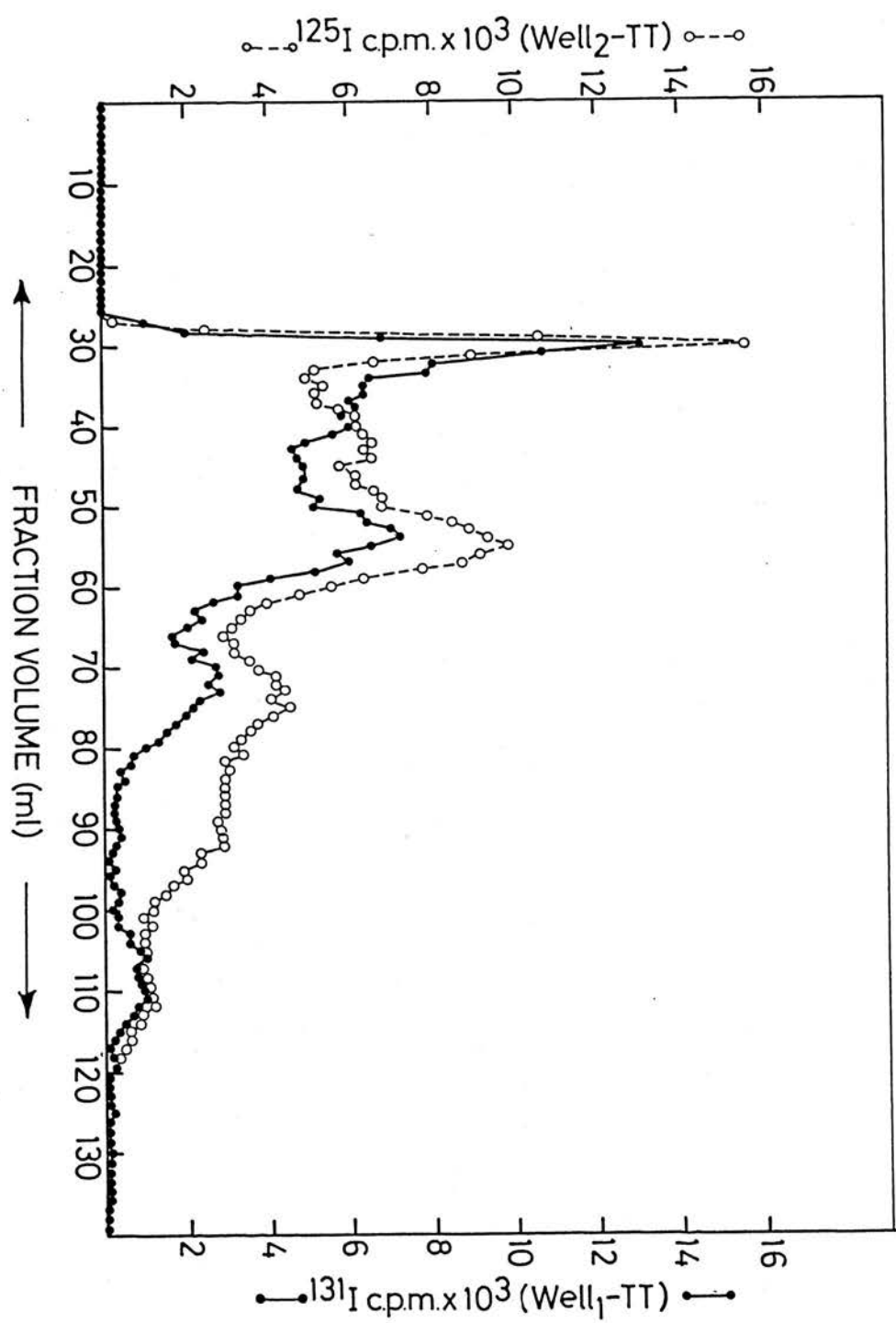


FIG. C.3-6: G-200 SEPARATION OF WELL<sub>1</sub>-TT ( $^{131}\text{I}$ -labelled) and WELL<sub>2</sub>-TT ( $^{125}\text{I}$ -labelled) COMBINED.



FIG. C.3-7

The tetanus toxoid preparation from a manufacturer in Canada (Conn-TT) was not only more concentrated with higher toxoid activity as compared to the other preparations (see also Table 1 & Fig. 8), it consists of only one main protein peak (o—o 4.5S) as superimposed on the NHS standard curve (●—●).

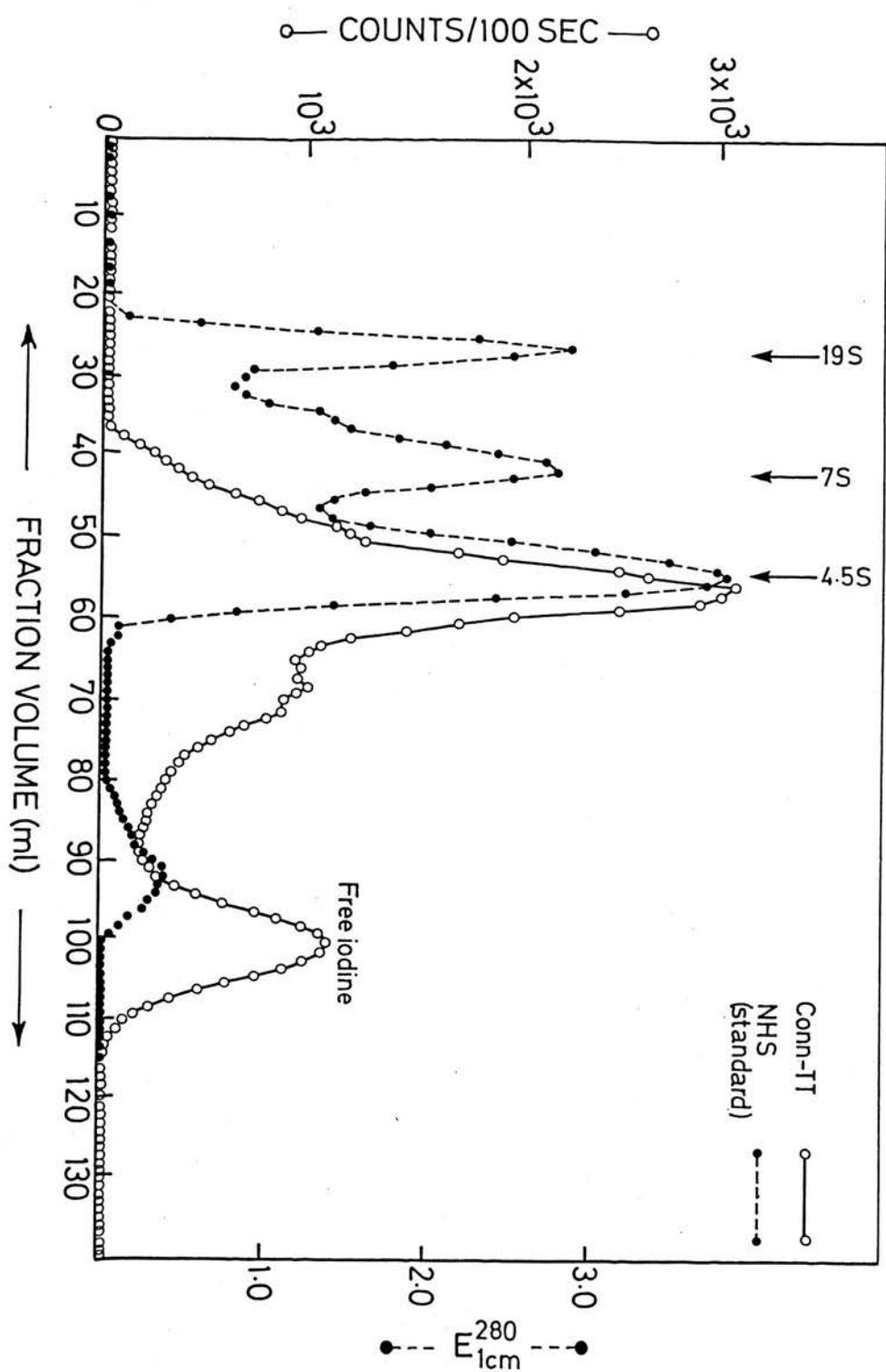
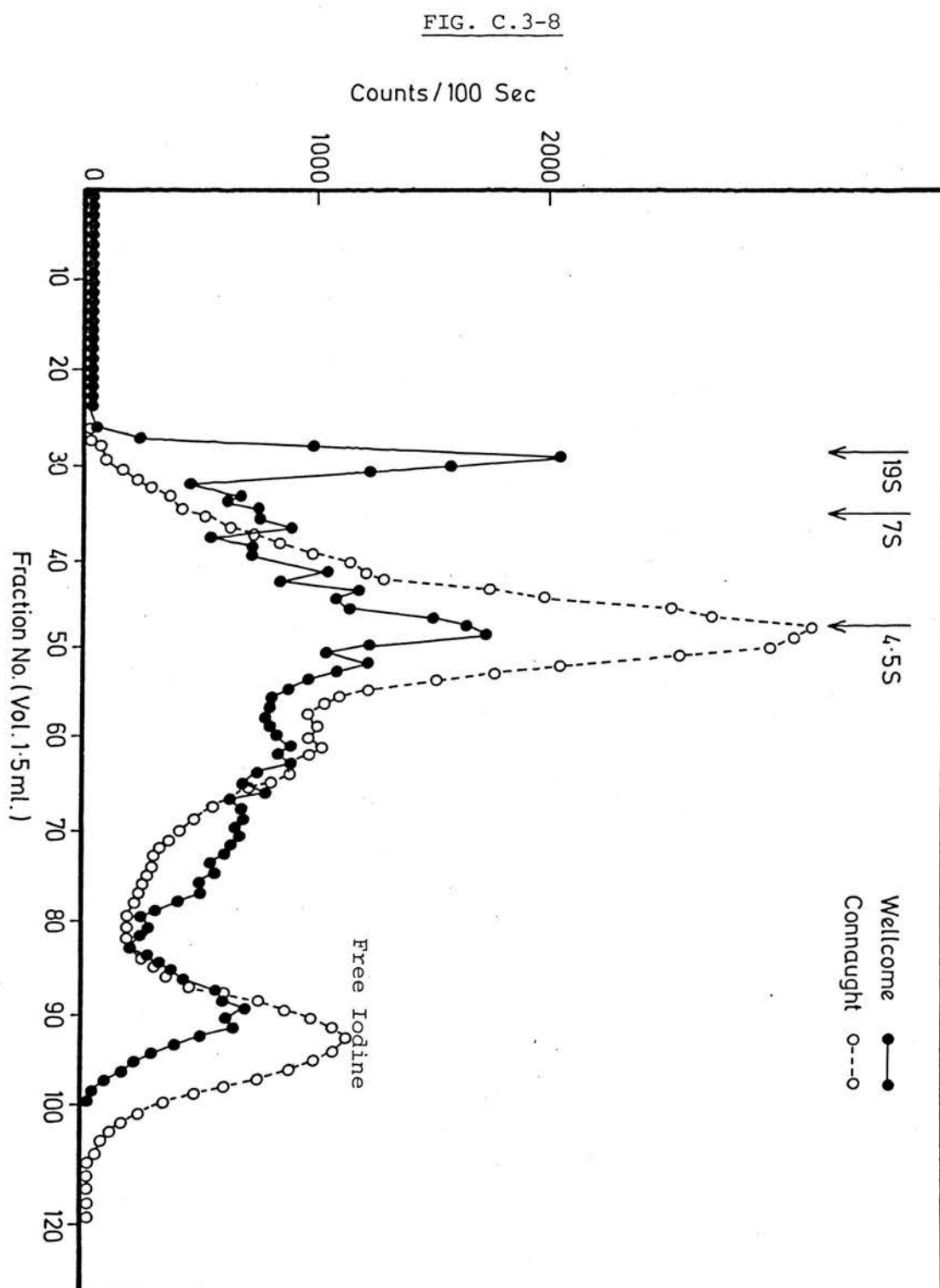


FIG. C.3-7: G-200 SEPARATION OF CONN-TT (TAS-112)

FIG. C.3-8

The superimposed elution profiles (G-200) of two tetanus toxoid preparations from different sources illustrate the heterogeneity of Wellcome vaccine (Well-TT ●—●) and the homogeneity of Connaught toxoid (Conn-TT ○—○). The 4.5S peak contained all of the toxoid activity (See Fig.4).



C.4 CHAPTER 4

THE RESPONSE OF MICE TO TETANUS TOXOID

#### C.4 THE RESPONSE OF MICE TO TETANUS TOXOID

##### 4.1 Introduction

The response of two strains of mice to two tetanus toxoid preparations was investigated partly because the study was of interest in itself and partly because it could yield information that would help our eventual aim of producing a suitable anti-tetanus antibody preparation for clinical use.

At the time these studies were initiated, anti-human isotype antisera for investigating the isotypes response in the human were not available. Furthermore it was difficult to obtain preimmunization samples in humans for investigating the primary response.

One point that we hoped to resolve was whether anti-tetanus responses were subclass restricted. In humans, responses to diphtheria and tetanus toxin have been variously reported as being predominantly IgG<sub>1</sub> (Yount et al., 1968), and involving all four subclasses (Carrel et al., 1972; van der Gissen et al., 1976). Subclass restricted responses to many antigens have been reported in mice (Torrighiani, 1972; Pelmutter et al., 1978; der Balion et al., 1980), and humans (Yount, 1968; Robby, 1970; Carrel et al., 1972; van der Gissen et al., 1976). Subclass restricted responses might be important in dictating recovery from infection and the therapeutic effectiveness of passively transferred antibody.

The study in mice was also performed to find out if different tetanus toxoid preparations varied in their immunogenicity and whether there was any major genetically determined difference in the immune response of two mouse strains. These were important preliminaries to the production of monoclonal antibodies to tetanus toxoid.

The anti-tetanus Ig-isotypes were assayed by a sandwich enzyme immunoassay (See Section C.1.4) which could be readily adapted to the human system, although unfortunately shortage of time prevented much work being done on the human Ig-isotype responses.

#### 4.2 Mice and immunization schedule

The experiments were performed in 10-12 week old male CBA/Ca and BALB/c mice. The CBA/ca mice were bred from breeding stock originally obtained from the MRC Laboratory Animal Centre (Carshalton, England). The original BALB/c breeders were supplied by Olac Ltd. (Bicester, England). The mice were housed under conventional conditions and fed on "libitum maintenance diet no.1" supplied by Special Diet Service Ltd. (Wilham, England).

The mice were injected with one of two tetanus toxoid preparations: Wellcome tetanus-toxoid (Well<sub>1</sub>-TT) and a purified form of tetanus toxoid (Conn-TT) from Connaught Laboratories, described previously (Section C.3). Mice were injected ip with 10 µg protein in phosphate buffered saline on days 0, 28 and 56. Six mice receiving each treatment were bled out on days 14, 28, 42, 56 and 70. Sera from uninjected mice served as preimmunization controls. Samples were tested by ELISA (Section C.1.3), and by SEI assay (C.1.4).

#### 4.3 Results

##### 4.3.1 A comparison of the response of CBA/ca and BALB/c mice to Well-TT

The responses of CBA/ca and BALB/c to Well-TT were almost identical (see Fig. 1 and 3). Sera from unimmunized mice of both strains contained antibodies to Well-TT but only of the IgM, IgA and to

a lesser extent IgG<sub>3</sub> isotypes. Up to 28 days after one injection of Well-TT there was no discernible increase in the production of anti-tetanus antibody of any isotype, with the sole exception of a slight IgG<sub>2a</sub> response by CBA/ca mice. Following the first booster injection anti-tetanus antibodies of the IgG<sub>1</sub>, IgG<sub>2a</sub> and the IgG<sub>2b</sub> appeared and with subsequent boosting some of these continued to rise but IgG<sub>1</sub> gave the highest titres. The isotypes that were represented in the pre-existing antibodies showed less dramatic increase after boosting and indeed the titres of IgM anti-tetanus antibodies remained virtually unchanged. This agrees with the data of Ersher et al. (1982) who also failed to find a very pronounced IgM antibody response of mice to tetanus toxoid.

#### 4.3.2 A comparison of the response of BALB/c mice to the different tetanus toxoid preparations

The responses of BALB/c mice to Well-TT and Conn-TT are shown in Figs. 2 and 3. Several differences were found. Pre-existing antibodies of the IgG<sub>3</sub> isotype to Conn-TT were not detected although IgM and IgA antibodies were present. The response to Conn-TT was prompter than that observed with Well-TT. IgG subclass responses to Conn-TT (except IgG<sub>2b</sub>) were detected following primary challenge and reached consistently higher levels.

#### 4.3.3 Investigation of the cross-reactivity of antibodies evoked by the different tetanus toxoids

In order to examine the possible cross-reactivity of Ig-isotype antibodies elicited by the different toxoids, the reactivity of the same sera from BALB/c mice that were tested in the preceding section



and raised against both toxoids were assayed in plates coated with either of the toxoid preparations. Similar levels of binding were found irrespective of the antigen used to coat the plates (see Figs. 4 and 5). This indicates that the differences noted in Figs. 2 and 3 are determined by the antigen used for immunization rather than the assay procedure.

These results also confirm the reproducibility of the assay system used in the present studies.

#### 4.3.4 Further studies on the nature of the pre-existing antibodies to tetanus

Additional experiments were performed to confirm the isotypes of pre-existing anti-tetanus antibodies in BALB/c serum. Affinity chromatography of the serum was done on protein A-Sepharose using the elution conditions indicated in Fig.6 (see also Section B.5). The fractions eluted with the various buffers were concentrated and analysed for anti-tetanus isotype using the Sandwich Enzyme Immunoassay procedure (Section C.1.4). The results (see Fig. 7a & b) confirmed that the pre-existing anti-tetanus antibodies were predominantly of the IgM and IgA isotypes.

#### 4.4 Discussion

Subclass-restricted responses to a variety of antigens have been found in mice (Torrigiani, 1972; Pelmutter et al., 1978; Der Balion, 1980) and man (Yount et al., 1968; Robby, 1970, van der Gissen et al., 1976). The reasons for selective expression are not clear. The antigens do not bear any obvious similarities in chemical or physical characteristics. Differences in antigen presentation may be involved

(Der Balion et al., 1980) as may regulating idiotypic networks (Geha, 1982). In man, the response to tetanus toxoid has been variously reported as being predominantly IgG<sub>1</sub> (Yount et al., 1968; van der Gissen, 1976) and unrestricted (Carrel et al., 1972). In this study the responses of two strains of mice to the Well-TT preparation showed no great restriction. The responses to Conn-TT were detected in all IgG subclasses except IgG<sub>2b</sub> after primary challenge.

The presence of pre-existing IgM and IgA antibodies to tetanus toxoid in mice of both strains is of interest. Since the majority of people are immunized against tetanus, few studies are available on pre-existing antibodies in man, although before the discovery of tetanus toxoid Tenbrock and Bauer (1923) reported the detection of antitoxin antibodies in the blood of one third of a group of inhabitants of Peking, China, whose stool contained tetanus bacilli. A more recent serological study in unvaccinated humans and animals in the Galapagos Islands by Veronesi et al. (1983) found varying titres of antibodies. No reports have been available on the Ig-isotype of naturally occurring anti-tetanus antibodies. The fact that the same titres of natural antibodies were detected against the purified tetanus toxoid as against the cruder preparation makes it likely that the antibodies were in fact against tetanus toxoid and not a contaminating protein. These possibilities could readily be tested in mice.

The most probable explanation for the existence of naturally occurring antibodies to tetanus toxoid in man and mice would be that the organism stimulates a response after it is swallowed. Veronesi and co-workers (1983) have even suggested that the oral route might provide an effective vaccination route for humans against tetanus. This route might preferentially stimulate IgM and IgA isotypes. Alternatively

determinants might be shared between tetanus toxoid and other macromolecules or intestinal organisms such as other Clostridium spp.

The finding that the responses of the mice to both tetanus toxoid preparations were largely in the IgG isotypes agrees with the report of Ershler et al. (1982) who found that mice produced a poor primary response to tetanus toxoid and on boosting the antibody was mainly IgG<sub>1</sub>. It seems possible that the naturally existing IgM and IgA antibodies may play a role in inhibiting the subsequent response of these isotypes.

The lack of a primary response to Well-TT appeared to be due to the crude nature of the preparations; it contains 60 times less toxoid activity than Conn-TT at the same protein concentration. Tetanus toxoid (here Conn-TT) appears in general to be highly immunogenic, eliciting a primary response in mice with low doses (Willcox, 1975, and this thesis). In spite of the crude nature of Well-TT no differences were found in the ability of the two preparations to bind antibody suggesting that responses to contaminating proteins are weak.

#### 4.5 Conclusions

1. Conventionally housed CBA/ca and BALB/c mice were found to have pre-existing antibodies to tetanus-toxoid.
2. These "naturally acquired" antibodies to tetanus toxoid were of the IgM, IgA and to a lesser extent of the IgG<sub>3</sub> isotypes.
3. CBA/ca and BALB/c exhibited similar responses to Well-TT. A significant rise in titre of any isotype not being detected until after boosting.
4. Conn-TT in the dose used, elicited a more rapid and pronounced response than Well-TT. Responses were detected in all IgG subclasses except IgG<sub>2b</sub> after primary challenge.

FIG. C.4-1

C.B.A. mice were injected with tetanus toxoid on days 0, 28 & 56 and sera were collected once every 2 weeks. The level of anti-tetanus Ig-isotypes was measured by SEI assay. Note the presence of pre-existing antibodies of IgM and IgA and to a lesser degree IgG<sub>3</sub> isotype. Antibody response to Well-TT is similar to the BALB/c mice (Fig.3). There is poor IgG-subclasses response after primary challenge with Well-TT, with the exception of IgG<sub>2a</sub>. Following booster injection IgG<sub>1</sub> gave the highest titre.

FIG. C.4-1: CHARACTERIZATION OF TETANUS-ANTIBODIES PRODUCED FOLLOWING THE IMMUNIZATION OF C.B.A. MICE WITH "WELL-TT"

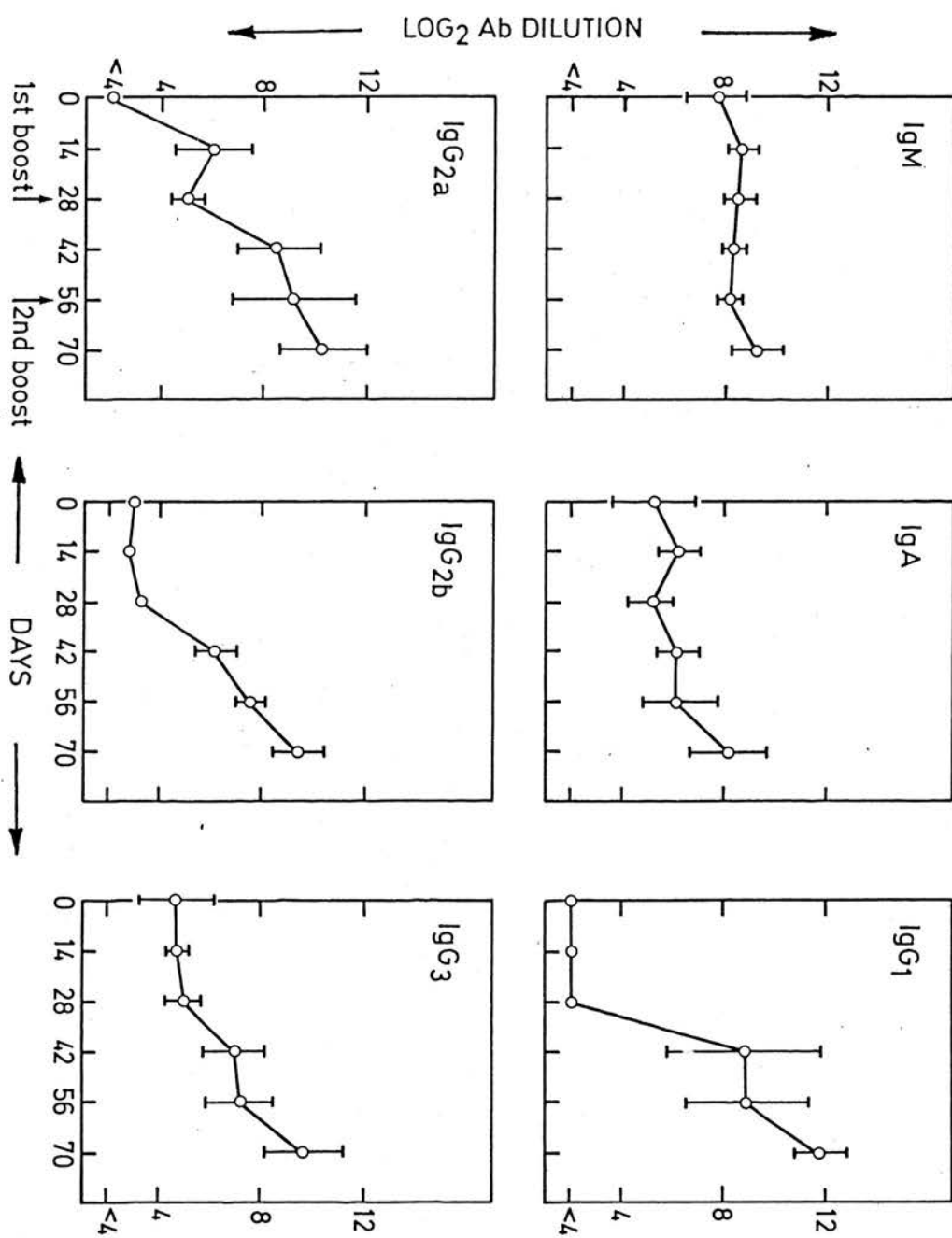


FIG. C.4-2

The Ig-isotype response of BALB/c mice to Conn-TT was prompter than that observed with Well-TT (Fig.3). IgG subclass responses was detected immediately following primary challenge with Conn-TT (with the exception of IgG<sub>2b</sub>), and reached consistently higher levels.

FIG. C.4-3

The pre-existing IgM and IgA antibodies were also seen in mice injected with Well-TT. But unlike the response to Conn-TT (Fig.2), IgG subclass responses to Well-TT was observed only following the boost.

FIG. C.4-2: CHARACTERIZATION OF TETANUS-ANTIBODIES PRODUCED FOLLOWING  
THE IMMUNIZATION OF BALB/c MICE WITH "CONN-7T"

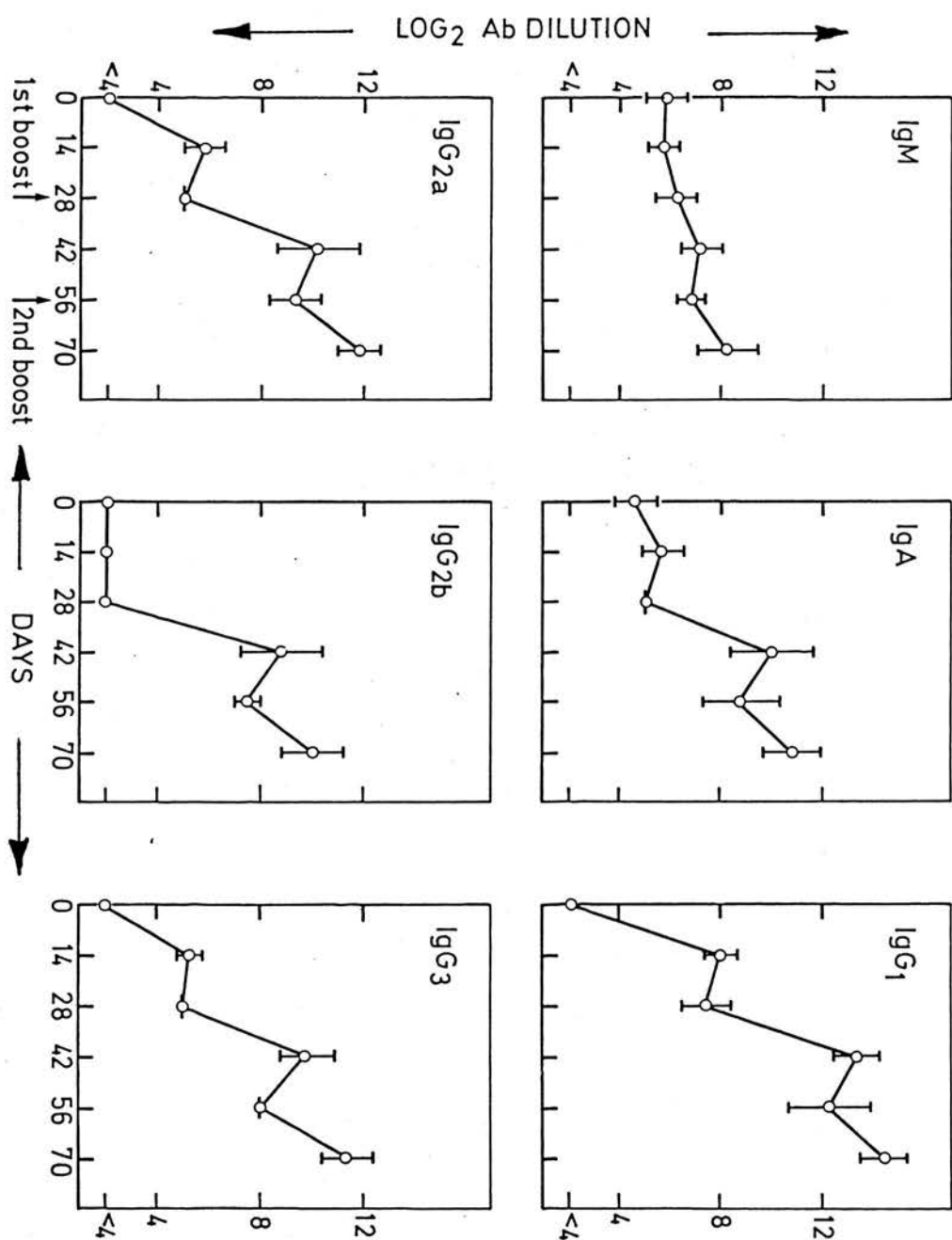


FIG. C.4-3: CHARACTERIZATION OF TETANUS-ANTIBODIES PRODUCED FOLLOWING THE IMMUNIZATION OF BALB/C MICE WITH "WELL-TT"

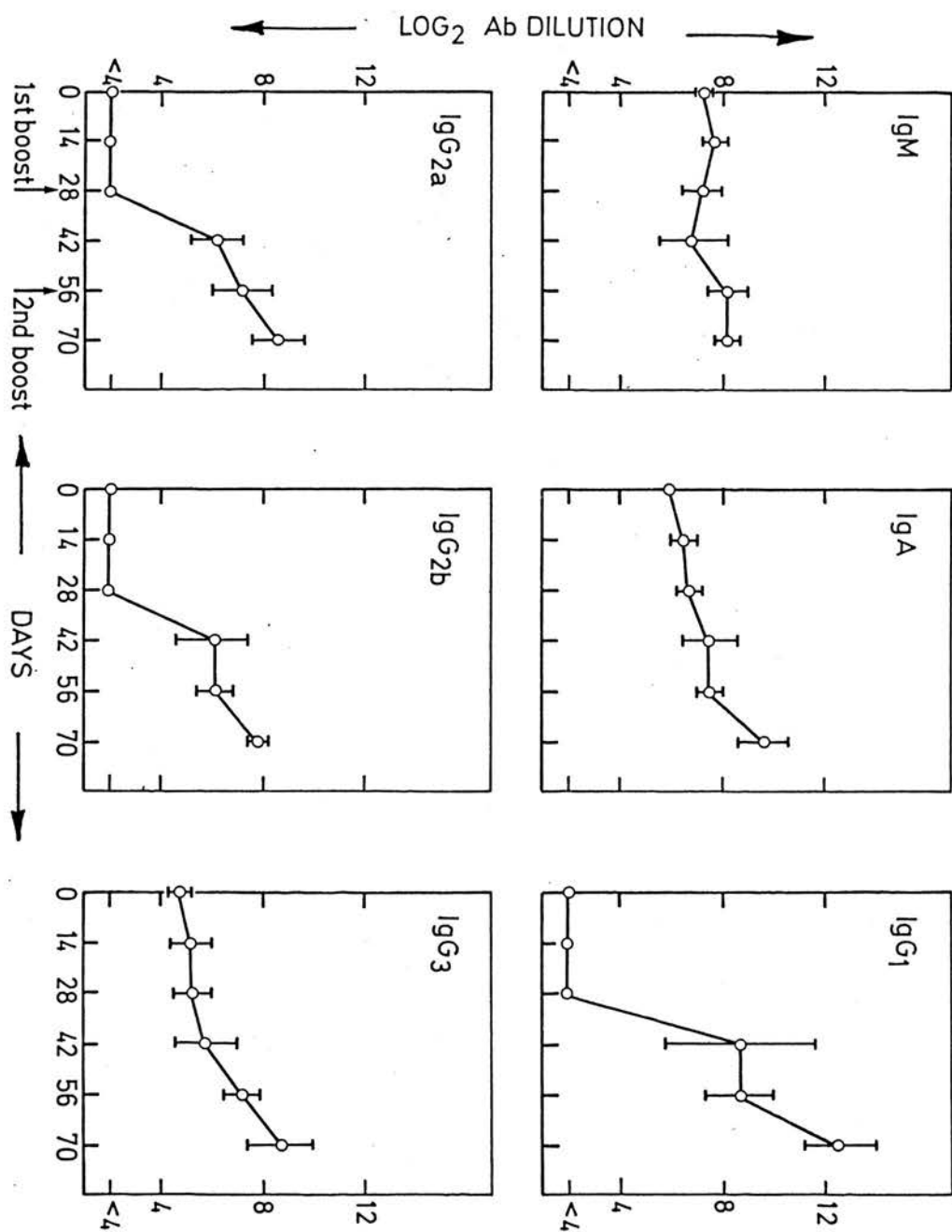




FIG. C.4-4

The cross-reactivity of Ig-isotype antibodies evoked by Conn-TT were measured by testing the antisera in plates coated with either of the toxoid preparations. Similar levels of bindings were found irrespective of the antigen coated to the plates (see also Fig.2).

FIG. C.4-5

The cross-reactivity of Ig-isotype antibodies were measured by testing the antisera from Well-TT immunized mice (BALB/c) in plates coated with either of the toxoid preparations. Similar results are observed as compared to Fig.3. These results (Figs. 4 & 5) indicate that the differences noted in Figs. 2 & 3 are determined by the antigen used for immunization rather than the assay procedure.

FIG. C.4-4: MICE Ig-ISOTYPE RESPONSE TO "CONN-TT" TESTED ON PLATES  
COATED WITH THE SAME TOXOID (●—●) or with "Well-TT" (○—○).

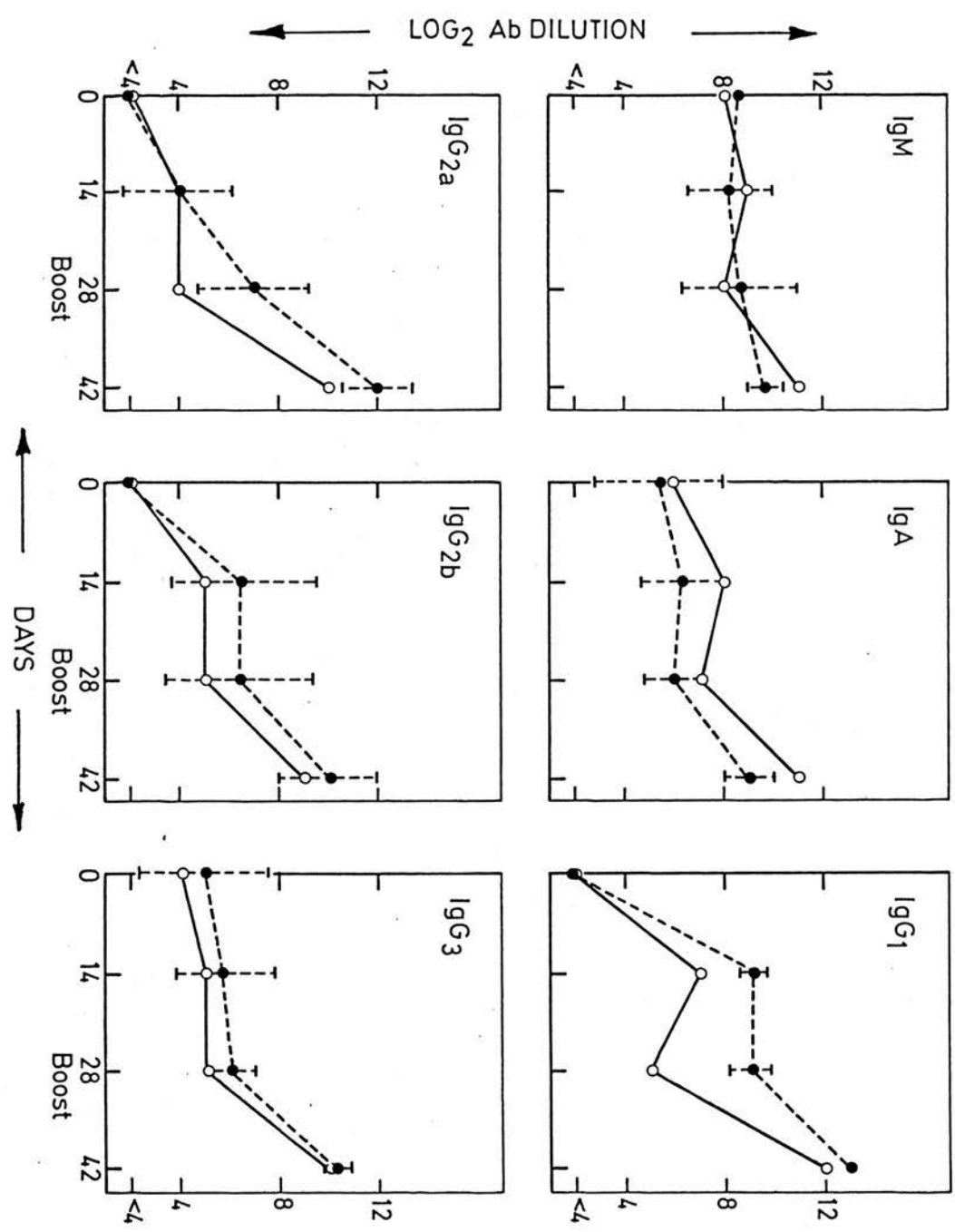


FIG. C.4-5: MICE Ig-ISOTYPE RESPONSE TO "WELL-TT" TESTED ON PLATES COATED WITH THE SAME TOXOID (●---●) OR WITH "CONN-TT" (○—○)

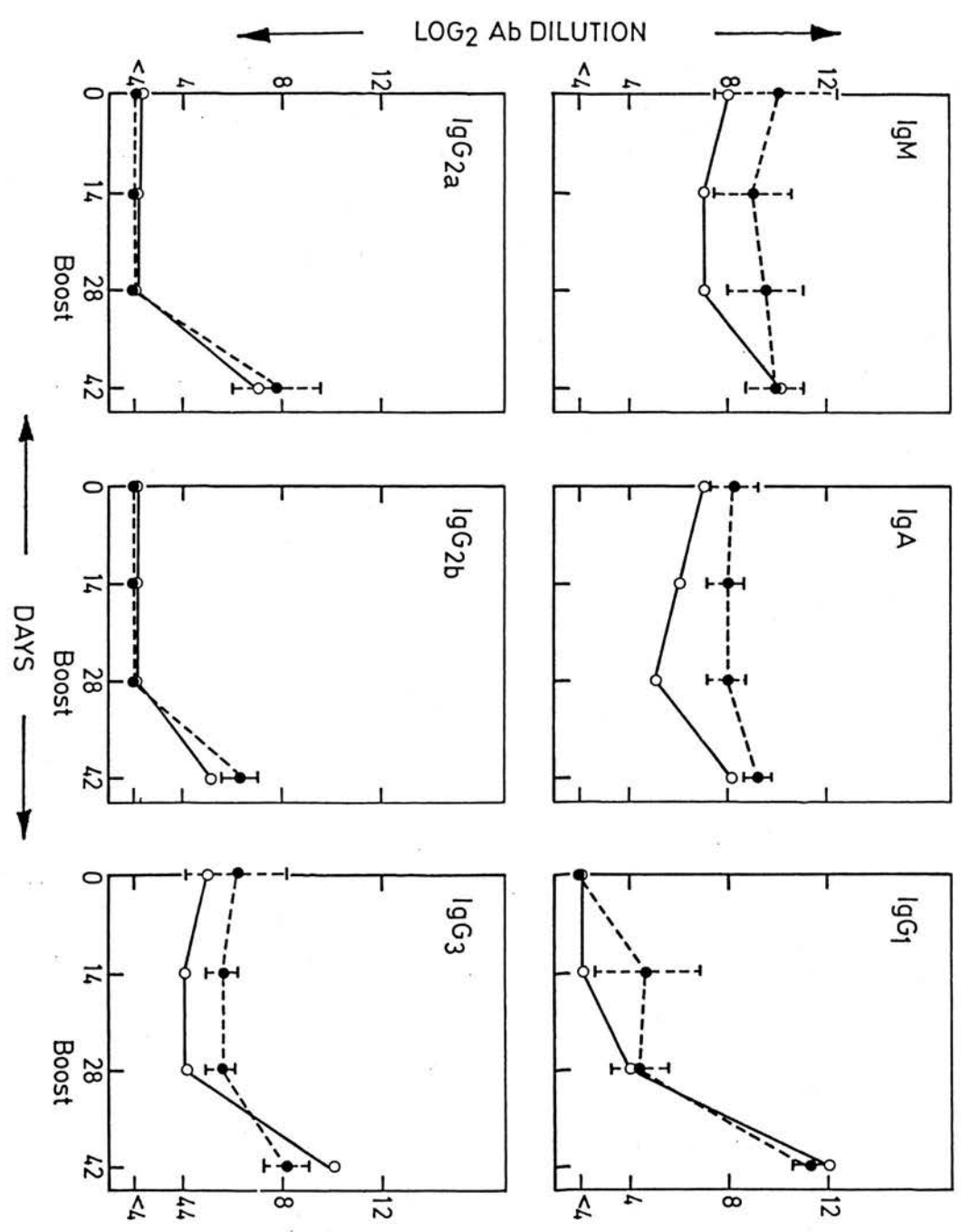
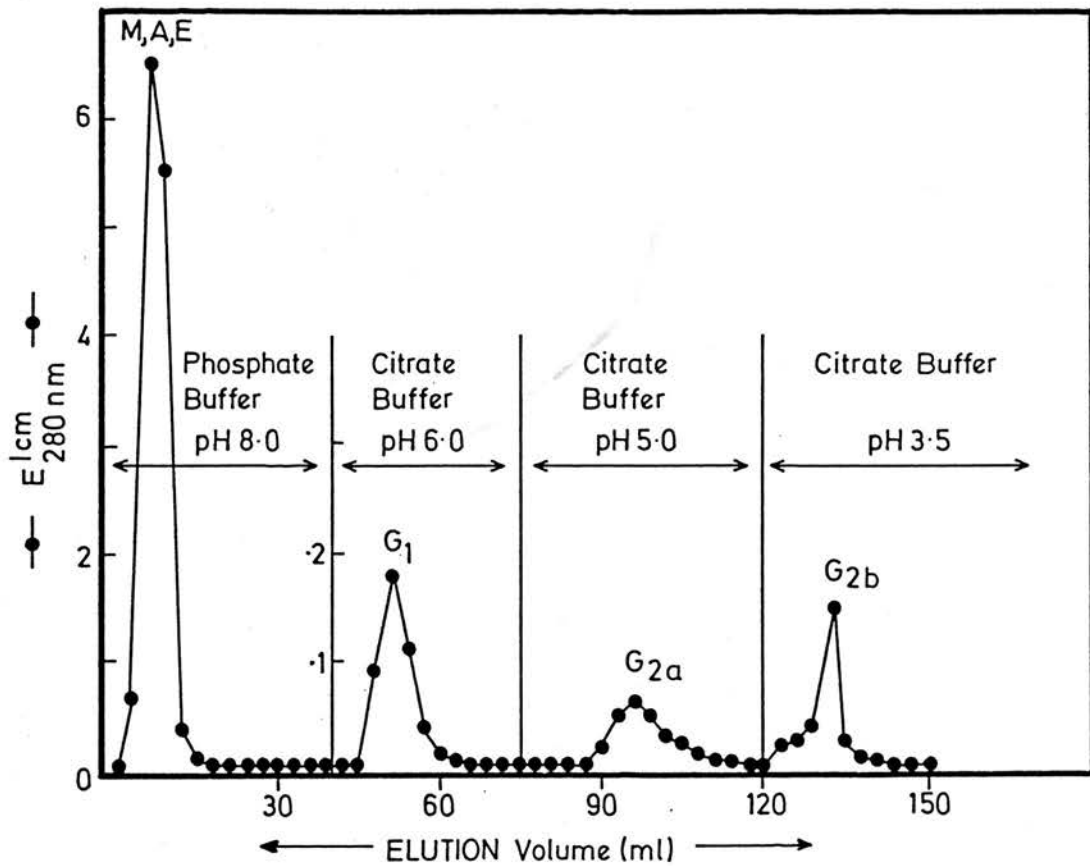


FIG. C.4-6

Ig - ISOTYPE SEPARATION OF NORMAL MOUSE SERUM (BALB/C)  
ON PROTEIN - A SEPHAROSE



Each peak was pooled and concentrated. The Ig-isotypes of the concentrated fractions were measured by SEI assay (see Fig. 7 a & b)

FIG. C.4-7a

The separated and concentrated Ig-isotype fractions of NMS (Fig. 6) were tested by SEI on "Well-TT" coated plates to investigate the nature of the pre-existing antibodies to tetanus. The results indicate that the pre-existing antibodies were predominantly of the IgM and IgA isotypes.

FIG. C.4-7b

Same concentrated fractions (Fig. 6) were tested on "Conn-TT" coated plates and these results also confirm that the pre-existing antibodies were mainly IgM and IgA.

FIG. C.4-7a: SEI RESULTS ON PROTEIN A SEPHAROSE FRACTIONS OF NMS  
(BALB/c) TESTED ON "WELL-TT" COATED PLATES

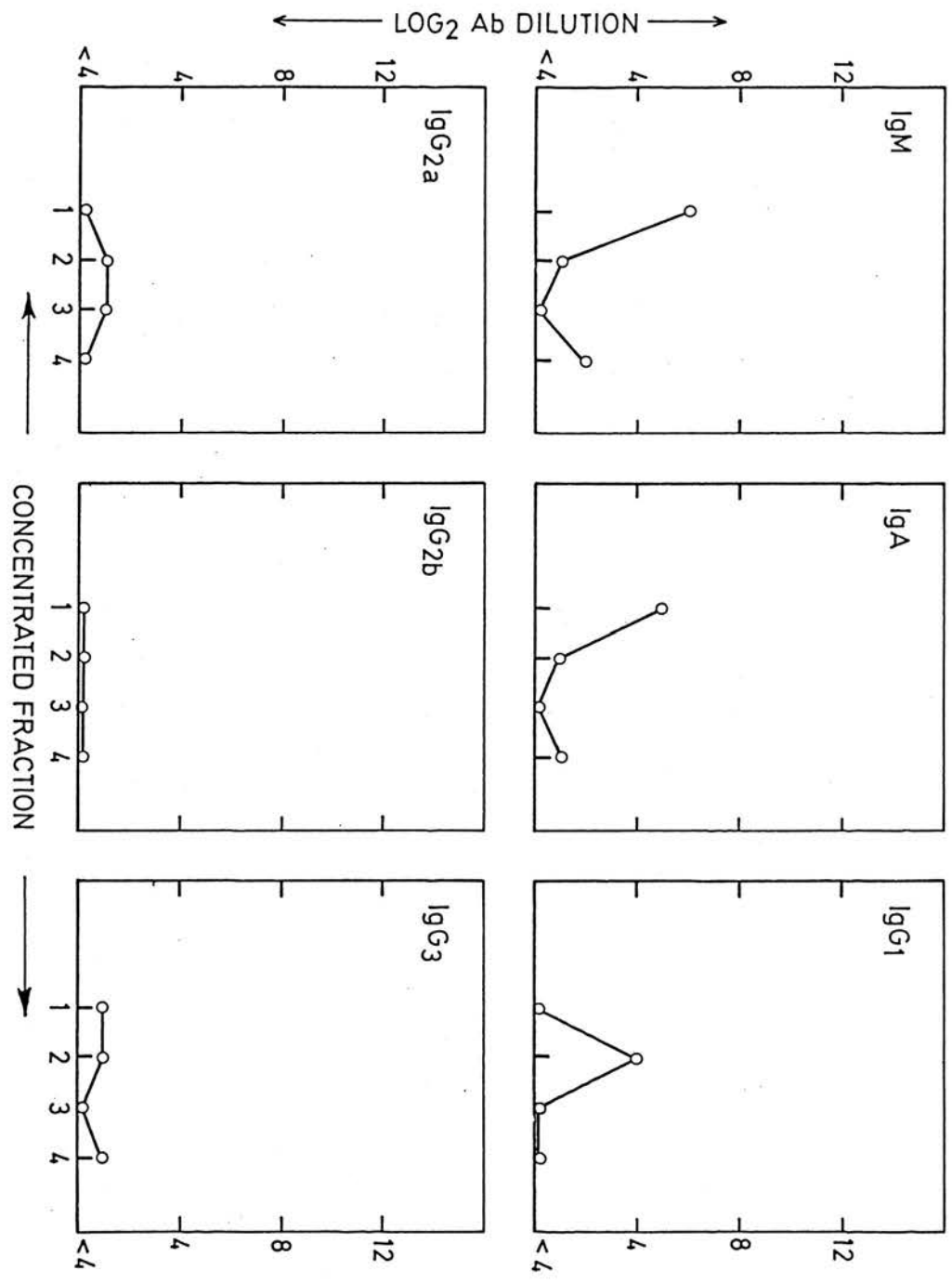
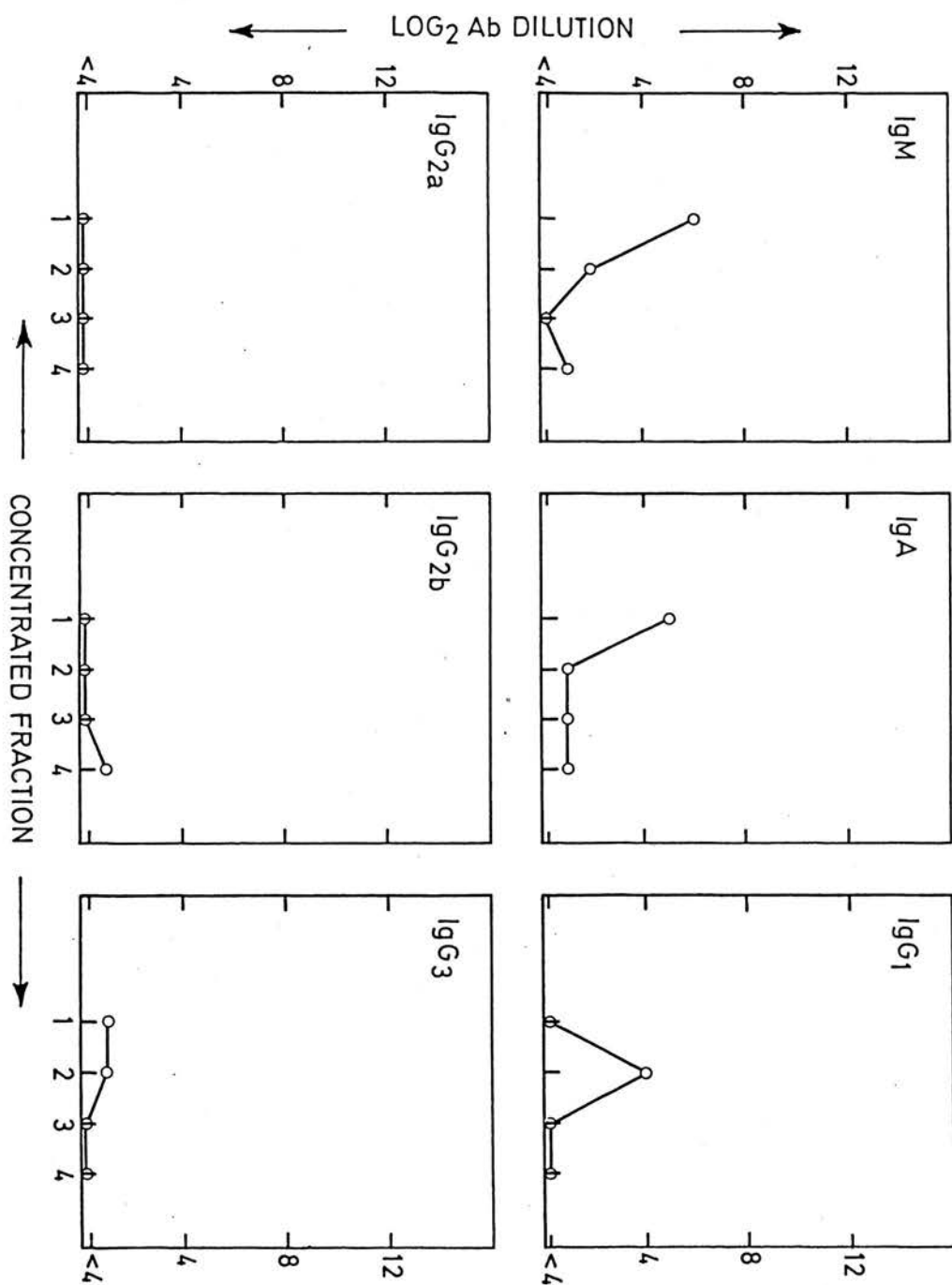


FIG. C.4-7b: SEI RESULTS ON PROTEIN A SEPHAROSE FRACTIONS OF NMS  
BALB/c) TESTED ON "CONN-TT" COATED PLATES



C.5 CHAPTER 5

IMMUNOAFFINITY PURIFICATION OF ANTI-TETANUS ANTIBODY



## C.5 IMMUNOAFFINITY PURIFICATION OF ANTI-TETANUS ANTIBODIES

### 5.1 Introduction

The use of human immunoglobulin preparations as a means of transmitting passive immunity is well-established. This approach is widely used in the prophylaxis of hepatitis A and B, varicella zoster, measles, vaccinia, rubella and tetanus, and it is of particular value in the management of patients whose own antibody producing ability is impaired by therapy or disease.

Conventional high titre immunoglobulin preparations for clinical use contain a very small proportion of specific antibody. They all contain large amounts of IgG which is not directed specifically against the relevant antigen. As a result, a large dose of protein must be given to patients to administer a small dose of the specific antibody. Large intramuscular injections are painful and the volume which can be given remains strictly limited. A further limitation on the use of these products is that intravenous administration often leads to adverse hypersensitivity reactions. Some of these problems may be overcome by the production of a purified immunoglobulin preparation of high specific activity so that it is possible to give a large dose of antibody in a small volume containing relatively little protein. Such preparations are likely to be safe to administer by intramuscular, intravenous and probably intrathecal routes.

Tetanus continues to be a major problem especially in the developing countries of the world. Until very recently the mortality rate reported from India and from other countries has been in the range of 22-58% (Thomas et al. 1982). Special surveys conducted in some developing countries have revealed that the disease was to be listed

among the ten principal causes of deaths of the population. Up to 10% of new-born babies were its primary victims (Bytchenko et al. 1981). A recent report published on anti-tetanus prophylaxis (Brand et al. 1983) indicates that one third of patients who contract the disease in the United States have either no obvious wound or a wound considered to be trivial by the patient. Men over 60 years of age and women over 40 are often not protected against tetanus, either because they have never been fully immunized or because they have not received the necessary booster doses of tetanus toxoid. The case-fatality rate of 45-55% in the United States has not changed since 1975. Their survey of six hospital emergency rooms reveals incorrect treatment. Persons most in need of adequate anti-tetanus prophylaxis were least likely to receive it (one patient in four). There has been over-treatment with tetanus toxoid, and under-treatment in conditions necessitating passive immunization with tetanus immunoglobulin. Analysis demonstrated the feasibility of improving the quality of care without adding to the cost of care.

The importance of passive immunity using tetanus-IgG, and increasing demand for a more purified and specific anti-tetanus antibody, prompted the work described in this thesis. In the initial stages, I concentrated on the isolation and characterization of this antibody from both whole plasma and Cohn fractionated IgG. The technique used was affinity chromatography which has a number of unique advantages over conventional methods. Affinity chromatography exploits the functional specificities of biological systems for the isolation of proteins, polysaccharides, nucleic acids and other classes of naturally occurring compounds. The unique specificity of biological interactions, for example antigen-antibody, provides entirely new

opportunities for achieving separations which are difficult or even impossible using less specific techniques.

The preparation of immobilized proteins and other materials requires an insoluble matrix to which the biopolymer can be attached without the loss of its specific binding properties. CNBr-activated Sepharose 4B, a bead-formed agarose gel displays nearly all the characteristics of the ideal matrix. It shows very little non-specific adsorption, has good physical, chemical and thermal stability and has good flow properties. The open pore structure of Sepharose 4B which allows it to be used for gel filtration of proteins up to 20 million daltons, enables large molecules to be coupled in good yield. The closed pore structure of cellulose and conventional polyacrylamide gels is distinctly disadvantageous (Boegman et al. 1970; Steers et al. 1971).

This section of the thesis will cover studies on (a) the preparation of immunoaffinity columns, (b) their application in antibody isolation and (c) the characteristics of the antibodies obtained by this procedure.

## 5.2 Optimization of binding

The basic procedure for preparing immuno-affinity columns is described in detail in Section B.6. The amount of tetanus toxoid bound to CNBr-Sepharose under various conditions was determined using  $^{125}\text{I}$  labelled tetanus toxoid as a tracer. In some of the experiments the amount bound was also determined by measuring amount of protein (both bound and free).

The influence of the following factors on binding was determined:-

- (a) Different antigen concentrations and sample volumes.
- (b) Different incubation periods and temperatures.
- (c) Batches of CNBr-Sepharose of different ages.
- (d) Different coupling buffers.
- (e) Reproducibility of coupling procedure.

#### 5.2.1 Antigen (TT) Concentration

5.2.1.1. Effect of sample volume: The details and results of these experiments are recorded in Table 1. It is apparent that the smaller the volume the better the binding (% coupled-TT is (a) > (b) > (c)).

5.2.1.2. Effect of amount of antigen: As shown in Table 2, both unconcentrated (1x) and 5x concentrated TT preparations coupled more efficiently to sepharose than 10x concentration. This may be due to CNBr-Sepharose capacity of binding being exceeded using 10x concentrated tetanus toxoid.

#### 5.2.2 Effect of incubation period and temperature

The % binding of tetanus toxoid to CNBr-Sepharose increased with time at room temperature (R.T.), but plateaued at 4 hours. At 4°C the coupling was more efficient than at R.T. up to 4 hours, but thereafter dissociation appeared to have occurred (see Table 3).

#### 5.2.3 Age of CNBr-activated Sepharose 4B

Tetanus toxoid was coupled to different batches of CNBr-Sepharose with different date of expiry. As shown in Table 4 the coupling

capacity of the older batches appears to have deteriorated.

#### 5.2.4 Coupling buffer

The coupling efficiencies of tetanus toxoid from two different manufacturers (Connaught and Wellcome) were compared using two different working buffers. In this experiment both % counts and % protein bound (TT) to CNBr-Sepharose was measured. As the results in Table 5 demonstrate, higher % binding was achieved using borate as the coupling buffer as compared to P.B.S. These results also showed that Conn-TT binds better to CNBr-Sepharose than does Well-TT. Binding, as assessed by radioactive counting was irreproducible. This might be due to free iodine, or loss of coupled gel in the filter during washes, since this irreproducibility was not observed in % uncoupled TT.

#### 5.2.5 Reproducibility of the coupling procedure

In order to determine the reproducibility of the coupling technique, a series of immunoabsorbent columns were prepared by coupling the  $^{125}\text{I}$ -labelled TT to CNBr-activated Sepharose 4B under precise conditions. Tables 6 and 7 show the results obtained with these separate batches. The coupling procedure is reproducible considering the many steps involved in the process. This reproducibility is very apparent if one looks at the % uncoupled-TT where the post coupling wash can easily be collected and measured (see Table 6).

#### 5.3 Conclusions of optimum conditions for coupling tetanus toxoid to CNBr-activated Sepharose 4B

According to results, binding is influenced by antigen concentration, temperature, incubation period, age of the sepharose

and the coupling buffer. Best results were obtained with:-

5.3.1 A higher concentration of TT in a smaller volume (minimum 1 mg/ml).

5.3.2 Incubation period of 4 hours at R.T. or overnight at 4°C.

5.3.3 Fresh batch of CNBr-activated Sepharose 4B.

5.3.4 Using 0.1 M borate buffer pH 8.0 as coupling buffer.

#### 5.4 Immunoaffinity purification of human anti-tetanus antibodies

##### 5.4.1 Materials and methods

The basic technique for coupling and preparation of the immunoabsorbent column has been described in details in Section B.6. In summary 3.5 mg of tetanus toxoid (Wellcome) was coupled to 2.5 g of CNBr-activated Sepharose 4B. Following equilibration the antibody containing plasma (20 mls) or Cohn-fractionated tetanus IgG (2.5 mls containing 250 I.U. antitetanus) was applied to the immunoabsorbent column. The unbound protein was then eluted with PBS 7.2. Fractions of 3 ml were collected using an automatic fraction collector until the optical density of the effluent reached zero. The specifically bound antibody was recovered by elution in 0.2M glycine-HCl pH 2.8. Fractions of 1.5 ml were collected in 0.5 ml of 2M Tris-HCl pH 7.8 to neutralize the eluate and minimise denaturation. The O.D.s of all fractions were measured in a Unicam SP500 spectrophotometer at 280 nm, and values were represented as  $E_{280\text{ nm}}^{1\text{ cm}}$

The distribution of anti-tetanus antibody activity (antibody titre) in the effluent was determined by passive haemagglutination assay (as in Section B.8), and ELISA (Section C.1.2), and expressed in IU/ml.

The eluates of each run were divided into four fractions and concentrated by ultrafiltration through 8/32 Visking dialysis tubing (as in Section B.2).

After determination of antibody activity by HA and ELISA, the total protein content (mg/ml) of the concentrated fractions was measured by the Folin-phenol procedure (Section B.1).

The concentration of IgG, IgA and IgM antibodies were determined by radial immunodiffusion (Section B.7) and expressed in mg/ml of the protein.

The specific activity and the % recovery of the specific antibody or IgG were calculated as follows:-

$$\text{Specific activity} = \frac{\text{Ab-titre of fraction}}{\text{IgG concentration of fraction}}$$

% Recovery =

$$\frac{\text{IgG concentration (or I.U.) of fraction} \times \text{volume}}{\text{IgG concentration (or I.U.) of original plasma (IgG)} \times \text{volume}} \times 100$$

#### 5.4.2 Results

Table 8 is merely a key to tube pooling of the effluents of each run. Results of two runs which are illustrated in Fig.1 and Fig.2, show that the affinity chromatography technique can be used to concentrate anti-tetanus antibody from human plasma (Fig.1) and human IgG concentrates (Fig.2). There is a higher concentration of

anti-tetanus antibody in glycine-HCl eluates compared to PBS eluates of the unbound proteins.

Table 9 is a summary of the results obtained on concentrated fractions. The specific activity is much higher in fraction B (specifically bound protein) as compared to fraction A (unbound protein). The bulk of the IgG and antibody activity (I.U.) were recovered in fraction B. In run 2 performed on anti-tetanus IgG, fraction B contained up to 96% of total anti-tetanus antibody activity in approximately 1% of the original protein. This fraction contained antibody of the IgG class only, with specific neutralizing activity of 151 I.U. per mg of IgG, representing 108-fold purification.

### 5.5 Discussion

Immunoabsorption is a useful method when low concentrations of antibody are to be isolated from plasma or from crude immunoglobulin fractions for prophylactic and therapeutic uses.

The coupling of the antigen was routinely performed at 4°C, and the elution at room temperature. The chemical stability of the immunoabsorbent column was excellent. Regular usage and washings with acidic buffer are possible without loss of capacity or specific properties, as long as the column is equilibrated with neutral buffer before storage at 4°C, between runs. Studies using <sup>125</sup>I-labelled tetanus toxoid indicates that loss of antigen from immunoabsorbent column following coupling is minimal. It was observed that the column capacity did not decrease with repeated use. The same column was used in all the four runs described without any variation in the results obtained.

The immunoaffinity chromatography can be used to recover the human specific tetanus antibodies in a highly purified and concentrated state



on an industrial scale. It can be carried out in a closed system and very rapidly.

Good results were obtained using this technique in order to concentrate anti-tetanus antibody from human plasma and human IgG concentrates. The specific activity obtained was about 80-182 I.U. per mg of protein (=52-108 fold purification), and 1% of the total IgG of the starting material contained up to 96% of the specific antibody activity. This indicates that the starting material contained large amounts of IgG which were not directed against the relevant antigen (tetanus toxoid).

Using this technique, monospecific antibody obtained could also be quickly isolated from human plasma with low antibody titre or from crude IgG preparations. Using such concentrated antibody preparations it would be possible to administer large dose of specific antibody with safety which is of considerable clinical importance.

The same technique could undoubtedly be applied to recover from donor blood antibodies against viral antigens such as: Hepatitis surface antigen, Cytomegalovirus, Herpes Zoster, Herpes Simplex, or bacterial antigens such as Pneumococcal polysaccharide.

#### 5.4.2 Conclusions of immunoaffinity purification

Good results were obtained using the immunoaffinity technique described to purify human anti-tetanus antibodies. The following conclusions are drawn.

5.4.3.1 Antibody of higher specific activity is obtained using this technique.

5.4.3.2 Can be used to enrich low titre antibodies in plasma.

5.4.3.3 The immunoaffinity columns can be re-used, which is important as regard to costs.

5.4.3.4 Unbound protein can be processed further.

5.4.3.5 The procedure is rapid and reproducible. It enables rapid preparation of highly specific antibody preparation of clinical value.

5.4.3.6 The technique is widely applicable.

TABLE C.5-1

SAME AMOUNT OF TT IN DIFFERENT VOLUME

Volumes (mls)  (TT 6.5 mg)	% Coupled in duplicate (4°C O/N)	
	A	B
(a) 3	25	37
(b) 6	16	28
(c) 12	11	11

It is apparent that the smaller the volume the better the binding of tetanus toxoid to CNBr-activated Sepharose (% coupled-TT is (a) > (b) > (c)).

TABLE C.5-2

SAME VOLUME BUT DIFFERENT AMOUNT OF TT

Concentration of TT (in 4.0 mls)	% Coupled (at 4°C O/N)
Conc. (10x) = 12.0 mg	40
Conc. (5x) = 4.0 mg	62
Unconc. (1x) = 1.2 mg	60

Both 1x and 5x concentrated tetanus toxoid coupled more efficiently to Sepharose than 10x conc. This may be due to CNBr-Sepharose capacity of binding being exceeded using 10x conc.

TABLE C.5-3  
EFFECT OF INCUBATION PERIOD AND TEMPERATURE

Incubation time  (TT conc. 2.5 mg/4 ml)	% Coupled TT	
	R.T.	4°C
1 hr	10	23
2 hrs	15	22
4 hrs	21	24
0/N	21	17

The % binding of tetanus toxoid to CNBr-Sepharose increased with time at R.T., but plateaued at 4 hrs. At 4°C the coupling was more efficient than at R.T. up to 4 hrs.

TABLE C.5-4

EFFECT OF AGE OF CNBr-SEPHAROSE ON COUPLING CAPACITY

Expiry date of CNBr-Sepharose	% Coupled TT (2.5 mg at 4°C O/N)
1 year (prior to)	39
1 month (post)	28
1½ year (post)	22
3 years (post)	16

The coupling capacity of the older batches of CNBr-Sepharose appears to have deteriorated.

TABLE C.5.5

COMPARISON OF THE COUPLING EFFICIENCY OF TT  
USING DIFFERENT WORKING BUFFERS

Buffers	TT (in duplicate)	% Uncoupled TT		% Coupled TT	
		Counts	Protein	Counts	Protein
0.1M Borate + 1M NaCl pH 8.0	Well-TT	19	25	17	75
	Well-TT	19	25	27	75
	Conn-TT	5	3	26	97
	Conn-TT	4	3	37	97
Stock Solution PBS pH 7.2	Well-TT	43	61	8	39
	Well-TT	44	60	15	40
	Conn-TT	16	16	24	84
	Conn-TT	18	17	5	83

Higher % binding was achieved using borate as the coupling buffer as compared to PBS. These results also show that Conn-TT binds better to CNBr-Sepharose than does Well-TT.

TABLE C.5-6

DETERMINATION OF THE REPRODUCIBILITY OF COUPLING PROCEDURE

USING WELL-TT

Well-TT (2.6 mg/4 ml)	% Uncoupled TT (Counts)	% Coupled TT (Counts)
1	27	42
2	28	43
3	26	40
4	28	47
5	27	44
6	27	34
7	27	23
8	26	20
9	27	19
10	28	17

The reproducibility of coupling procedure is apparent if one considers the many steps involved in the process. This reproducibility is particularly shown in the % uncoupled-TT, where the post coupling wash can easily be collected and measured (see also Table 7).



TABLE C.5-7

DETERMINATION OF THE REPRODUCIBILITY OF COUPLING PROCEDUREUSING TWO DIFFERENT TT

TT Samples (3.5 mg/4ml)	% Uncoupled TT		% Coupled TT	
	Counts	Protein	Counts	Protein
Well-TT				
1	16	19	55	81
2	18	20	54	80
3	17	19	53	81
4	22	24	49	76
5	22	28	47	72
Conn-TT				
1	8	6	57	94
2	6	5	49	95
3	8	5	67	95
4	9	6	58	94
5	9	6	59	94

These results confirm the reproducibility of the coupling technique, especially if binding is assessed by the protein measurement. By counting the radioactivity of the antigen, the % coupled-TT is much lower. This might be due to free iodine or loss of coupled gel in filter during washes.

TABLE C.5-8

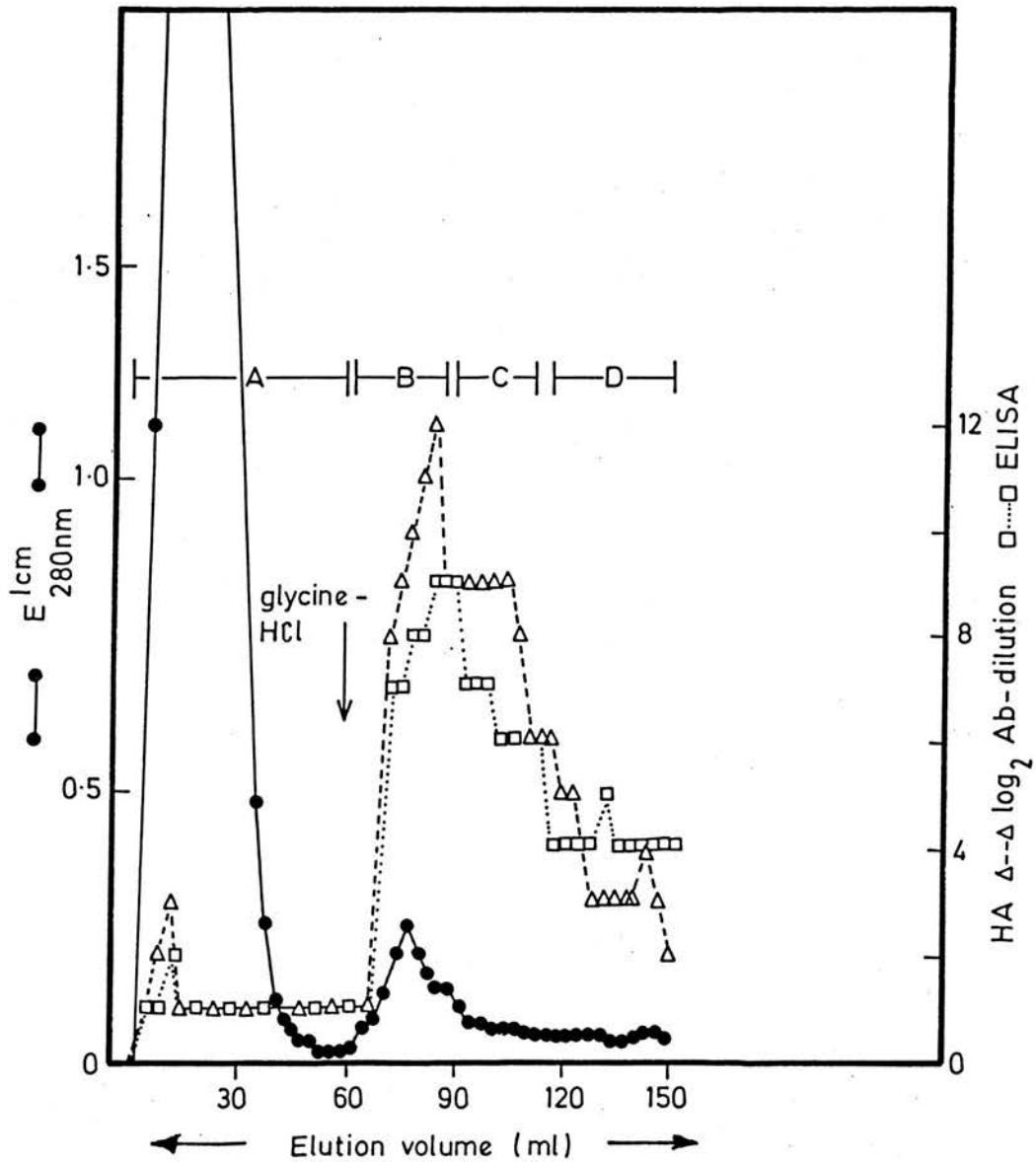
KEY TO TUBE POOLING

Run	Fraction	Washing Buffer	Tubes
Serum 1	Fr. A	PBS pH 7.2	1 - 20
	Fr. B	Glycine-HCl pH 2.8	21 - 30
	Fr. C	"	31 - 38
	Fr. D	"	39 - 50
Serum 2	Fr. A	PBS pH 7.2	1 - 17
	Fr. B	Glycine-HCl pH 2.8	27 - 36
	Fr. C	"	37 - 44
	Fr. D	"	45 - 56
Anti-tetanus IgG 1	Fr. A	PBS pH 7.2	1 - 14
	Fr. B	Glycine-HCl pH 2.8	37 - 47
	Fr. C	"	48 - 52
	Fr. D	"	53 - 60
Anti-tetanus IgG 2	Fr. A	PBS pH 7.2	1 - 16
	Fr. B	Glycine-HCl pH 2.8	17 - 28
	Fr. C	"	29 - 38
	Fr. D	"	39 - 50

The eluates of each immunopurification run (see Figs. 1 & 2) were divided into 4 concentrated fractions and were analysed extensively (see Table 9).

FIG. C.5-1

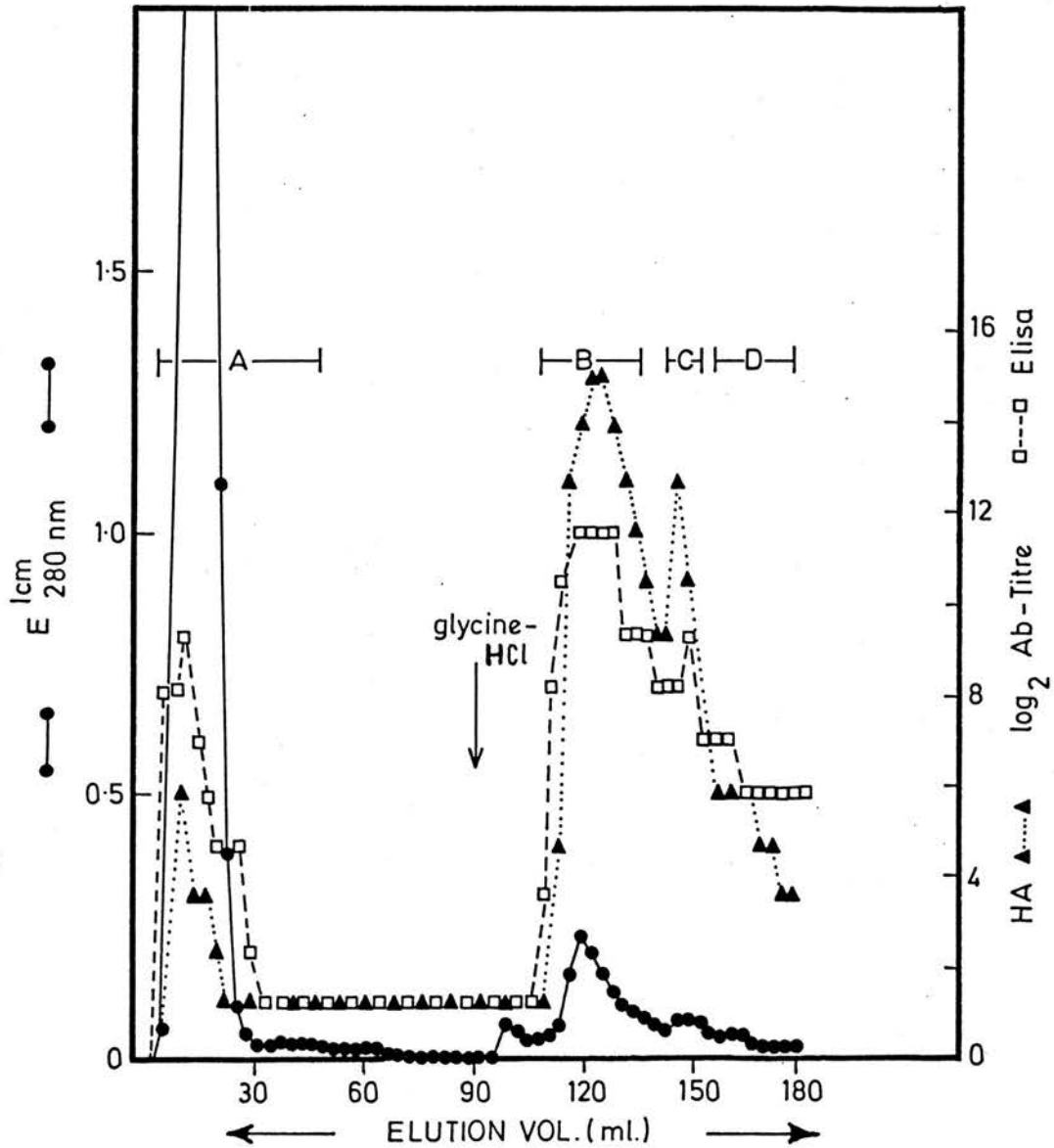
IMMUNO AFFINTY OF ANTI TETANUS ANTIBODIES ON A TETANUS - TOXOID -  
Cn Br-SEPHAROSE COLUMN (STARTING MATERIAL ANTITETANUS SERUM)



Anti-tetanus antibodies were isolated from the plasma of immunized donor. The specific antibody activities were assessed by HA ( $\Delta$ - $\Delta$ ) and by ELISA ( $\square$ - $\square$ ). There is a higher concentration of tetanus antibody in glycine-HCl eluates in much smaller amount of protein ( $\bullet$ - $\bullet$ ) compared to PBS eluates (the unbound protein).

FIG. C.5-2

IMMUNO AFFINITY OF ANTI TETANUS ANTIBODIES ON A TETANUS -  
TOXOID - Cn Br SEPHAROSE COLUMN (STARTING MATERIAL - TETANUS IgG)



Higher concentration of tetanus antibodies in smaller amount of protein was obtained by immunopurification of Cohn-fraction II (TIG). This is shown by the distribution of antibody titres and protein levels in the effluents.

TABLE C.5-9

A SUMMARY OF RESULTS OBTAINED ON CONCENTRATED FRACTIONS

Run	Sample	Vol. mls.	Protein Concentration mg/ml				Antibody titre		IU/ml		Specific Activity IU/mg IgG	Recovery		Concentration Factor (Fr.B)
			Total	IgG	IgA	IgM	ELISA	HA	ELISA	HA		IgG	I.U.	
1	Serum	20	45	6.9	2.02	0.71	1/2560	1/2560	20	20	2.9	100	100	52x
	Fr.A	10	72.8	10.62	3.7	1.31	40	40	0.16	0.16	0.02	77	0.4	
	Fr.B	2.7	1.02	0.2	-	-	5120	10240	20	40	150	0.4	20.3	
	Fr.C	2.6	0.7	-	-	-	160	160	2.5	2.5	-	-	0.41	
	Fr.D	2.4	0.72	-	-	-	640	640	2.5	2.5	-	-	1.5	
2	Serum	20	45	6.9	2.02	0.71	2560	2560	20	20	2.9	100	100	63x
	Fr.A	6.0	50.8	8.0	2.64	1.0	40	40	0.16	0.16	0.02	34.8	0.24	
	Fr.B	3.8	0.9	0.11	-	-	5120	10240	20	40	182	0.3	28.5	
	Fr.C	3.5	0.8	-	-	-	320	160	1.25	0.63	-	-	0.9	
	Fr.D	3.0	0.8	-	-	-	320	160	1.25	0.63	-	-	0.75	
1	Anti-Tet IgG	2.5	135	72.6	2.1	0.34	3200	3200	100	100	1.4	100	100	57x
	Fr.A	4.5	42.8	17.7	0.7	0.07	2560	2560	10	10	0.56	44	18.0	
	Fr.B	2.0	1.5	1.0	-	-	20480	20480	80	80	80	1.1	64	
	Fr.C	3.5	1.12	-	-	-	160	320	0.63	1.25	-	-	1.4	
	Fr.D	5.5	0.8	-	-	-	160	320	0.63	1.25	-	-	2.2	
2	Anti-Tet IgG	2.5	135	72.6	2.1	0.34	16000	8000	100	100	1.4	100	100	108x
	Fr.A	5.5	27	17.7	0.7	0.08	640	640	5.0	2.5	0.21	53.6	8.3	
	Fr.B	3.0	1.2	0.53	-	-	20480	20480	80	80	151	1.0	96	
	Fr.C	4.2	1.0	0.08	-	-	640	2560	2.5	10	78.3	2.3	10.5	
	Fr.D	3.5	1.06	-	-	-	320	1280	1.25	5.0	-	-	4.4	

Immunological and some biochemical properties of the separated fractions (of 4 immunoaffinity runs) are summarised. The bulk of IgG and anti-tetanus antibody activities were recovered in Fr.B (bound protein) as compared to Fr.A (unbound protein). Specific neutralising activities of 80-182 IU per mg of IgG were obtained depending on the specific activity of the raw material.

C.6 CHAPTER 6

STUDIES ON MONOCLONAL ANTI-TETANUS ANTIBODIES

## C.6 STUDIES ON MONOCLONAL ANTI-TETANUS ANTIBODIES

### C.6.1 Introduction

The generation of cell lines capable of permanent production of specific antibody directed against a predefined immunogen was first reported by Köhler and Milstein in 1975. This method was based on fusion between myeloma cells and spleen cells from suitably immunized animals. Antibody-producing lymphoid cells have a very short life when cultured under in vitro conditions, but individual myeloma cell lines can be grown permanently in culture. When both types of cells are fused, hybrids can be derived which retain the essential properties of (a) permanent growth and (b) production and secretion of antibody with a pre-defined specificity. Since the hybrid cells can be cloned, it is possible to direct the heterogeneous response of an animal (Fig.1a). The procedure therefore permits the derivation of monoclonal antibodies (McAb) directed against very well defined antigenic determinants, regardless of the complexity of the immunogen.

This discovery launched a new era in immunological research, and it allows a new strategic approach to a wide variety of problems. Hybridoma production represents a powerful tool for uniform, large-scale production of homogeneous antibody against a variety of antigens. Such reference reagents can be made in virtually unlimited amounts for indefinite periods of time. Since the hybridoma technology, in essence, clones out of the spleen individual antibody-forming cells, animals can be immunized with impure antigens and pure antibodies can be obtained. In principle, hybridoma can be developed which will produce a desired antibody of any given class, subclass, allotype, or even against a given antigenic determinant or

epitope. Monoclonal antibodies of these types have numerous potential investigational uses. This may conveniently be considered under the broad headings of diagnosis, treatment and prophylaxis. It would already be impracticable to list all of the monoclonal antibodies of clinical interest. They are already very widely used in clinical chemistry, clinical immunology, bacteriology, pathology and haematology (reviewed by McMichael et al. 1982 and James et al. 1984). In particular, they are now used for the routine typing of blood cells, analysis of lymphocyte subsets (James et al. 1984), the quantitation of a variety of drugs (Habor, 1982), hormones and serum components (NIAID study, 1981), the identification of microbial organisms (Mitchison et al. 1982 and Cohn, 1982) and in imaging and therapy of tumours where an antibody-drug conjugate (magic bullet) may be used (Greaves et al. 1982 and Thorpe et al. 1982).

Mouse monoclonal antibodies have already proved to be extraordinarily powerful new reagents in laboratory investigations. These tailor made reagents have improved the specificity, sensitivity and reliability of a number of in vitro diagnostic procedures such as radioimmunoassays, immunohistological and other immunoassays. Monoclonal antibodies have already provided probes for studying the structural-functional interrelationships of individual domains of the immunoglobulin molecule, in determining subtle conformational changes, and in studying V-region structure and genetics (NIAID study, 1981).

They have also advanced the applicability of immuno-affinity purification techniques for isolation of biologically important macromolecules from recombinant and other sources, or the removal of undesirable contaminants (reviewed by James et al. 1984).

During recent years, monoclonal antibodies have played an



important role in monitoring therapeutically important substances and as therapeutic agents. They have made possible the identification of functionally different subpopulations of lymphocyte. The relative proportions of T cell-subsets seem to be an important determinant of immune status (James et al. 1984), and the antibodies can be used in the pre-treatment of homograft recipients (Kaplan et al. 1982).

Monoclonal antibodies are likely to have their greatest role in the study and treatment of viral illnesses, for which presently available antibiotics are known to be ineffective. These include: influenza, parainfluenza (Gigliotti et al. 1982), herpes (Ewing et al. 1979), measles, retrovirus, rabies and RNA tumour viruses (NIAD study, 1981). Using monoclonal antibodies, it has been possible to identify substrains of many of these viruses, indistinguishable with polyclonal antibodies.

Monoclonal antibodies may also be therapeutically useful in the management of infectious diseases caused by bacterial, fungal and parasitic agents. In addition to their direct neutralizing or inactivating effects on such agents, monoclonal antibodies of appropriate specificity can be conjugated to antibiotics and then targeted to achieve significantly higher local antibiotic concentrations, while reducing antibiotic levels in unaffected tissues (Kaplan et al. 1982).

We have attempted to produce continuous cell lines (human and murine) secreting monoclonal antibodies against tetanus toxoid. Tetanus toxoid was chosen as a model antigen because of its direct relevance to the other studies in this thesis on polyvalent anti-tetanus antibodies, and because of the obvious therapeutic potential of monoclonal anti-tetanus antibodies. Two types of

monoclonal antibodies were produced, initially of murine and later of human origin. The human monoclonal antibody was produced mainly by Epstein-Barr virus (EBV) transformation of antibody-producing lymphocytes derived from tetanus immunized donors. These lymphocytes were also fused with mouse myeloma cells to produce mouse-human, and with human lymphoblastoid cells to produce human-human hybridomas secreting human monoclonal antibody against tetanus.

The choice of assay used during the screening stages, to detect and clone the hybrid secreting the desired antibody, is of the utmost importance and should be given the greatest attention. There are many ways of detecting the presence of antibodies, but not all assays are directly applicable to monoclonal antibodies. First the concentration of antibody in the culture supernatant is usually much lower than that of hyperimmune serum, which makes the sensitivity and the specificity of the assay of great importance, and second because of the vast numbers of samples to be tested, and the urgency of knowing the results, so that the chosen hybrids can be quickly cloned, rapidity, simplicity and cost become very important. Taking these factors into consideration, I developed the ELISA and SEI techniques (described in Section C.1) to monitor the level and specificity of the antibody, and to determine the Ig-isotype of the monoclonal antibodies produced against tetanus. These techniques were used to (1) measure the anti-tetanus antibody levels of TT-immunized mouse and human, (2) monitor both murine and human fusion products and cloned cell lines, (3) assess the level of anti-tetanus antibody in ascitic fluid, (4) characterize the Ig-isotype of the monoclonal antibodies by determining the distribution of antibody (i.e. the isotype) in protein A-Sepharose fractionated ascites. In this Section the results obtained from these

studies will be discussed.

## 6.2 Production of hybridoma in mouse system

The cell lines making antibody against tetanus toxoid were produced according to the general methods of Köhler et al (1975) and Galfre et al. (1977).

Twelve week old BALB/c mice were immunized with 10 µg tetanus toxoid (Well-TT or Conn-TT) intraperitoneally (i.p.) in complete Freund's adjuvant. They were boosted (i.p.) with the same amount of the antigen in Freund's complete adjuvant on various days thereafter (see Table 1). The spleens were removed and splenic lymphocytes were suspended at a concentration of  $5 \times 10^7$  cells per ml in complete medium RPMI 1640 buffered with 20 mM Hepes, and supplemented with 2 mM glutamine, 1 mM sodium pyruvate, 50 units/ml penicillin, 50 µg/ml streptomycin, 10% foetal calf serum (FCS) and  $5 \times 10^{-5}$ M 2-mercaptoethanol. The spleen cells were fused with a non-secreting mouse myeloma cell line NS-1 abbreviated from P3-NS1/1-Ag 4-1 (Köhler et al, 1976). One week before the fusion, the myeloma cells were grown into 75 cm<sup>2</sup> flasks and were fed with the complete medium daily.

The method used for this fusion was as described by Oi et al. (1980), which was modified after Fazekas et al. (1980). In two of the fusions the T-cells were removed by running the spleen cells through a nylon-wool column (leukopack), and using the non-adherent cells for fusion. The fusion ratio in all experiments was two myeloma cells to one spleen cell. The fused cells were plated out in 96-Well culture plates,  $2.0 \times 10^5$  cells being added to each well, which contained a feeder layer of  $2 \times 10^5$  mouse thymocytes. Polyethylene glycol 4000 (BDH Chemicals) at 38% (W/V) concentration was used as the fusing agent. The fused cells were grown in RPMI 1640 medium (as described

but without mercaptoethanol), containing  $10^{-4}$ M hypoxanthine,  $4 \times 10^{-7}$ M aminopterin, and  $1.6 \times 10^{-5}$ M thymidine (HAT) (Littlefield, 1964). After seven days, aliquots of the supernatants were removed and tested for tetanus antibody activity by the ELISA procedure described in Section C.1.3.

Positive cultures were expanded and then cloned by limiting dilutions (Mishell et al. 1980) into 96-Well plates in presence of  $5 \times 10^5$  mouse thymocytes feeder cells per well. Clones were selected on the basis of both their antibody secreting capacity and their growth characteristics. As the clone cells multiplied, they were transferred from the 96-well culture plate to a 24-well plate, at which stage aminopterin was omitted from the medium (HT) and then into 25 cm<sup>2</sup> flasks with ordinary medium (without HT).

Antibody-secreting clones were also grown up in ascites form as follows: cells were harvested from cultures, resuspended in Dulbecco's Minimal Essential medium, and  $2 \times 10^6$  cells in 0.2 ml were injected i.p. to a number of BALB/c mice which had been injected i.p. with 0.5 ml Pristane (tetramethylpentadecane) ten and three days previously (Potter, 1972). Ascites developed in about seven days. The fluid was withdrawn from the abdominal cavity over a period of several days, and tested for antibody activity by the ELISA procedure.

The immunoglobulin isotypes of the monoclonal antibodies were analyzed by fractionation of the ascitic fluids on Protein-A Sepharose column according to the procedures described in Section B.5. The results of this procedure were subsequently confirmed by SEI assay (Section C.1.4) employing isotype specific antisera.

A summary of the steps involved in the production of mouse hybridoma producing monoclonal antibody is outlined in Figure 1a.

### 6.3 RESULTS (mouse cell lines)

#### 6.3.1 The production of mouse hybridoma secreting monoclonal antibody to tetanus toxoid

Over 3000 cultures were set up giving rise to large number of hybrids. Although some fusions resulted in high numbers of hybrids (see Table 1), the incidence of specific antibody-secreting hybrids was low. Table 1 (fusion 5 & 6) also shows that depletion of T-cells from spleen cells before fusion had little or no effect on the number of specific antibody-secreting hybrids.

#### 6.3.2 The production of anti-tetanus antibodies by growth of cell lines in ascites form

From fusion 1 (Table 1), two cell lines designated Fl.1 and Fl.6 continued to grow and produce monoclonal antibody to tetanus.

In an attempt to obtain anti-tetanus antibodies preparations of high titre, some of the antibody-secreting cell lines ( $10^7$  cells) were grown in ascites form. Studies in a large number of mice revealed that the yield of antibodies by this procedure was extremely variable even in mice challenged with the same cell line. However on occasions ascites of relatively high titre were obtained. Furthermore in these and other mice, the levels of anti-tetanus antibody appeared to increase in the later tapplings (Tables 2,3, and 4). The antibody activity in ascitic fluid of mice injected with uncloned cell lines (Fl.1 and Fl.6) was much lower (Tables 2 and 3) than in those injected with cloned cell lines. (Tables 4 and 5). When sequential tapping was performed, more concentrated antibody was obtained, and in most cases the Ab-titre increased linearly with subsequent tapping (Fig. 2 and

Table 4).

In a study carried out in the Department of Biochemistry (Van Heyningen), ascitic fluid with high titre of antibody (from cell line Fl.1.2) were found to bind to both chains of tetanus toxin.

#### 6.3.3 Purification and Ig-isotype characterization of the mouse monoclonal antibodies against tetanus

Although some of the ascites fluids obtained contained more than 40 times the antibody activity (Table 4) observed when the same cells were grown in culture, these preparations also contained large amounts of irrelevant protein of mouse origin. In order to isolate the monoclonal antibody, and identify its Ig-isotype, the ascites fluid was therefore fractionated on a protein A-Sepharose column. The results of two such fractionations are shown in Figs. 3 and 5. Almost all of the tetanus antibody activity was observed in the IgG<sub>1</sub> peak (Figs. 3 and 5). Further analysis on these fractions with isotype specific antisera using SEI assay confirmed that the antitetanus antibody was mainly of the IgG<sub>1</sub> class (Figs. 4 and 6). These studies also illustrate the effectiveness with which the various Ig-isotypes can be separated on protein A-Sepharose (see earlier studies on immune mouse sera, Section C.4).

#### 6.4 Production of human monoclonal antibody to tetanus

Whole blood from immunized volunteers was collected in EDTA. The volunteers had been immunized seven days or three months previously with 150  $\mu$ g in 0.5 ml of tetanus toxoid (Well-TT). The peripheral blood mononuclear cells were isolated by Ficoll-Hypaque gradient centrifugation (Böyum, 1968). The remaining procedures are outlined in

Fig.1b.

Two methods were used to prepare tetanus antibody-secreting cell lines of human origin; hybridization and viral transformation. Hybridization partners were murine plasmacytoma NS-1 (Köhler et al. 1976) and two clones (HMY 2.3 and HMY 2.9) derived from the human lymphoblastoid line LICR-LON-HMY 2 (Edwards et al. 1982). Lymphocytes were fused at a ratio of 2:1 using 35% (w/v) polyethylene glycol 1500 in serum-free medium for eight minutes.

In the viral transformation procedure antigen-specific lymphocytes were enriched by incubating the tetanus antibody-producing B cells on TT-coated plastic flask. The adherent B cells were subsequently recovered by adding lidocaine (4 mg/ml w/v) and were vigorously pipetted. The transforming B95-8 substrain of EBV from a marmoset cell line was used to immortalize the isolated B cells (Miller et al. 1973). EBV is a herpes virus that infects the primate B cell. Lymphocytes ( $10^6$ /ml) were incubated for 2 hours in supernatant from the EBV-producing cell line. The lymphoblastoid cell line so produced were treated in the same way as the hybrids.

After fusion or transformation, the cells were maintained in HAT medium as described in the mouse system. However on this occasion this medium was supplemented with 20% FCS, 1 mM oxaloacetate, and the antibiotics used were 10  $\mu$ g/ml Kanamycin 2.5  $\mu$ g/ml Fungizone. The cells were cultured initially at a density of  $2 \times 10^5$  cells/0.2 ml in flat-bottomed 96 well-microtitre plate, and in some of the experiments in 24 well-plates containing a feeder layer of  $5 \times 10^3$  mouse peritoneal macrophages.

For in vitro antigen boosting, mononuclear cells were incubated at  $2 \times 10^6$ /ml for 72 hours at 37°C in complete medium containing 5 ng/ml

TT and  $5 \times 10^{-5}$ M 2-mercaptoethanol, but omitting the HAT components.

Antibody-secreting cell lines were cloned by limiting dilution in U-bottomed microtitre plates in complete medium containing 30% thymocyte culture medium (TCM) prepared from BALB/c and CBA mouse thymocytes (Reading, 1982).

Culture supernatants were screened for anti-tetanus antibody activity by the ELISA procedure (as described in Section C.1.2)

The mouse toxin neutralization assay was performed by Dr. F. Sheffield at the National Institute for Biological Standards and Control (Holly Hill, London). The in vivo protection of anti-tetanus antibody secreted by one of the human cell lines, designated ES-12 was investigated in a standard 4-days mouse protection assay (British Pharmacopoeia, 1980). Culture supernatant from ES-12 had been tested without prior concentration or purification; culture medium from a non-secreting cell line acted as a negative control.

#### 6.4.1 Human Ab-response to TT-booster immunization

Healthy adult human volunteers were each given one intramuscular injection of Well-TT (0.5 ml). Blood samples were collected on 5, 7, 14 and 26 days, and at 6 months after the injection. The levels of anti-tetanus antibody in the samples were measured by both the ELISA and HA procedures.

### 6.5 RESULTS (human cell lines)

#### 6.5.1 Comparison of fusion partners

Fusion of human lymphocytes with the human lymphoblastoid HMY 2 line yielded more cultures than fusion with mouse myeloma NS-1 cell



line, under identical conditions (Table 7). HMY 2.3 appeared to fuse more successfully than HMY 2.9 line. That is, they gave rise to more hybrids. All cultures except those from HF2 fusion ceased to grow. In this fusion, where 480 wells had been seeded, only 55 wells showed cell growth, and three of these secreted tetanus-specific antibody. One of these cultures designated ES-12 has continued to grow and secrete anti-tetanus antibody.

#### 6.5.2 In vitro boosting with antigen

Pre-incubation of lymphocytes with TT before fusion or virus infection did not seem to increase either the number of wells with growth or the proportion secreting specific antibody (Table 7).

The immortalized ES-12 cell line which secreted TT-specific antibody was derived from a donor who had been boosted in vivo three months earlier and without further in vitro antigen stimulation before fusion.

#### 6.5.3 Preliminary characterization of tetanus antibody secreted by the ES-12 cell line

As shown in Table 7, only one heterohybridoma (using NS-1 as fusion partner) secreted antibody to tetanus as detected by ELISA. Although the antibody level was initially high, it declined rapidly and was undetectable 30 days after the fusion. Immediate cloning of the positive culture showed no antibody activity in the supernatants (ELISA results not listed). The cell line ES-12 was one of the five human-human cell lines producing high levels of antibody activity. In both the mouse (in vivo) protection assay and in vitro assay (ELISA), ES-12 had a titre of 2.5-3.0 I.U./ml as compared to reference human

standards (listed in table of antisera, and discussed in Section C.1.2). This cell line which continued to grow and secrete antibody to TT up to 9 months has been characterized further (Boyd et al. 1984 and James et al. 1984). Their results indicate that ES-12 cell line expresses only the HLA phenotype of the donor and secretes an IgG<sub>3</sub> kappa antibody. Its model chromosome number is 44, suggesting that it is a virus-transformed lymphoblastoid cell line and not a hybrid.

In an attempt to stabilize the cell line, ES-12 has been hybridized with an HAT sensitive lymphoblastoid cell line KR4 (Boyd et al. 1984). The new hybrid (HF25) has a model chromosome number of 86, it grows at a faster rate than the parent cell ES-12, but the level of IgG it secretes, and especially its anti-tetanus activity, is reduced (Boyd et al. 1984).

The antibody secreted by ES-12 cell line was shown to bind to both chains (H & L) of tetanus toxin in a study which has been carried out in the Biochemistry Department of Edinburgh University (Van Heyningen et al., personal communication).

#### 6.5.4 Human response to TT-booster immunization

The results given in Table 8 indicate that the anti-tetanus antibody levels before booster immunization were between 0.06 - 1.0 I.U./ml. The antibody levels then increased gradually up to day 14 after the booster immunization, and individuals achieved a significant antibody rise (16 - 32 I.U./ml) by this time. This high level of antibody was maintained in the donor's serum up to 6 months. The lymphocytes of donor 1 were collected 3 months after the boost, and these were used to produce the human monoclonal antibody to tetanus. At this time the donor had produced a 267-fold increase in the serum

concentration of specific antibody (Table 8, donor 1).

The results in Table 8 also demonstrate that the ELISA technique is more sensitive than HA. Negative controls using unimmunized human serum failed to give positive results.

## 6.6 Discussion

We believe that we have established continuous mouse and human cell lines capable of producing monoclonal antibodies to tetanus toxoid.

A large amount of mouse monoclonal antibody to tetanus was generated by growing the hybridoma as an ascitic tumour in BALB/c mice. Sequential tapping of ascitic fluid was performed to obtain more concentrated antibody, and in most instances the Ab-titre increased with the subsequent tapping. There are clearly advantages in using late tapplings for the preparation of monoclonal antibodies. It has been shown by other workers as well (Hunter et al. 1982) that Ab-titres vary from cell line to cell line and from mouse to mouse. As experienced in the Department of Surgery, it is best to grow ascites tumour in as many mice as possible and test the sequential bleed. Another advantage of serial tapping (K.James et al., unpublished) is that frequently much stronger and purer immunoglobulin of the specific isotype has been observed at the later taps.

The level of antibody activity of one of the two cell lines was low, and on occasions dropped to zero. It is apparent that the hybrid has either lost the relevant gene or has been overgrown by non-antitetanus-secreting hybrids. In such an instance it is vital to go back and regrow the original hybrid and reclone. Decrease or loss of secreted antibody has been reported by others (Zurawski et al. 1978)

and has been attributed to multiclonality. Cloning results in monoclones of identical cells. Should single cells subsequently lose one or more chromosomes, but remain viable and continue to divide, the original monoclonal culture will become biclonal (or even multiclonal). The new cell line with fewer chromosomes is different from the first, and that is one of the common reasons for loss of production from an originally high-producing clone. In those mice where ascites tumour did not grow, it is also possible that the numbers of cells injected were less than  $10^7$ . With fewer cells, the mouse may be able to defend itself against the tumour.

The ELISA assay proved to be highly sensitive, and allowed a rapid and reliable screening system of the hybrid cultures in both mouse and human systems. The dominant Ig-isotype produced by the two continuous mouse cell lines is IgG<sub>1</sub>, which is the isotype found in the majority of mouse monoclonal antibody (Lutz et al. 1983). Recently IgG<sub>1</sub> was found to be the predominant subclass of in vivo and in vitro produced anti-tetanus toxoid antibodies (Stevens et al. 1983).

Monoclonal antibodies to TT have been produced in murine (Butler, 1983) and human system (Chiorozzi, 1982; Gigliotti, 1982; Kozbar, 1982). However if they are to be considered for therapeutic use, it is important that they have protective activity in vivo. We were able to obtain a number of clones producing human anti-tetanus antibody. One of these, designated ES-12, has continued to grow and secrete specific antibody. Its culture supernatant without further concentration or purification has been shown to be protective against tetanus toxin in mice. In both in vivo protection and in vitro assay, the culture supernatant has a titre of 2.5 - 3.0 I.U./ml. This ES-12 cell line was obtained from fusion of immune lymphocytes with HAT-sensitive human

lymphoblastoid cell line (HMY 2.3). One of the problems involved in using lymphoblastoid cell line as a fusion partner in hybridization is that spontaneous EBV transformed cells can arise in culture. Most of the initial characterization of the ES-12 line was to determine whether it was a hybridoma or not (Boyd, Farzad et al. in press, and Boyd et al. 1984). On the basis of karyotype analysis and tissue typing studies, it would appear that the ES-12 cell line has arisen as the result of spontaneous transformation in culture of an EBV positive antibody secreting plasma cell. Studies on ES-12 cell line have been continued after the termination of my study (Boyd et al. 1984). In order to preserve the antibody secretion, ES-12 has been hybridized with KR4 lymphoblastoid cell line. Although the new hybrid has grown at a faster rate than the parent cell line ES-12, the amount of IgG produced and its anti-tetanus antibody activity has reduced. The ES-12 secrete IgG, kappa and like mouse McAb it binds to both chains of tetanus toxin (as tested by Simon van Heyningen) which agrees with other studies (Gigliotti, 1982).

The frequency of the human-mouse hybrids was very low, and they tended to be less stable than their human-human counterparts. Nevertheless, other groups have successfully produced mouse-human hybrids secreting anti-tetanus antibody (Gigliotti et al. 1982). The frequency of EBV-transformation of B-lymphocyte was relatively high, and high percentage of wells with growth showed anti-tetanus activity. The necessity for removing T-cells from the lymphocyte population to be fused is unclear, as conflicting reports have appeared in this area (Butler et al. 1983 and Gigliotti et al. 1982). In our own experience, T-cell depletion did not increase the efficiency of mouse fusion, but was essential for successful EBV transformation.

While preselection of antigen-specific lymphocytes can increase the proportion of antibody-secreting EBV-transformants (Kozbor et al., 1981), it is not yet certain whether this approach is necessary in the formation of hybridomas. Although initially there was a vigorous growth of EBV-transformed cell lines, which produced high levels of specific antibodies, they ceased to grow after a short time, and also proved difficult to clone, possibly due to loss of chromosomes. In contrast to other studies (Butler et al. 1983), in vitro boosting with TT prior to fusion did not seem to increase the TT-specific cultures by either the cell hybridization or EBV-transformation procedure.

The complexity of the interaction between variables can lead to contradictory results between laboratories. For example, it is conceivable that the beneficial effect of feeder cells is much greater if the foetal calf serum and media are suboptimal (Hurrell, 1982). Since there is no entirely satisfactory test to predict the suitability of the FCS, it is not easy to prove that the need for feeder cells, for example, are related to the FCS batch. Experiments to study all these variables are tedious and relatively uninteresting at a time when most investigators are anxious to produce some useful antibodies, irrespective of the efficiency of the process.

While the advent of monoclonal antibody technology offers many advantages, it must be stressed that it also suffers from a number of limitations. These include: (1) the excessive amount of work and time involved in generating and stabilizing monoclonal antibodies, (2) the difficulty in obtaining monoclonal antibodies against antigens that are poorly immunogenic, (3) their exquisite specificity can limit their use as therapeutic agents, rendering them less attractive at fixing complement, opsonizing bacteria, and clearing toxin than their

polyvalent counterparts and (4) the potential presence of EBV or oncogenic virus in antibody preparations.

However, with the recent advances in this technology and with the help of fast developing improvements in the cloning of specific antibody genes, the future for human monoclonal antibodies holds great promise.

## 6.7 Conclusions

Mouse and human cell lines secreting anti-tetanus antibody were produced.

6.7.1 The mouse monoclonals were grown in both tissue culture and ascites form. By performing sequential taps of ascites-bearing mice, anti-tetanus antibody preparations of high titre were obtained.

6.7.2 The antibody produced by both in vitro culture and in ascites was of the IgG<sub>1</sub> isotype.

6.7.3 A relatively stable human cell line (designated ES-12) secreting anti-tetanus antibody was obtained by fusing PBL from a TT-immunized volunteer with a human lymphoblastoid cell line.

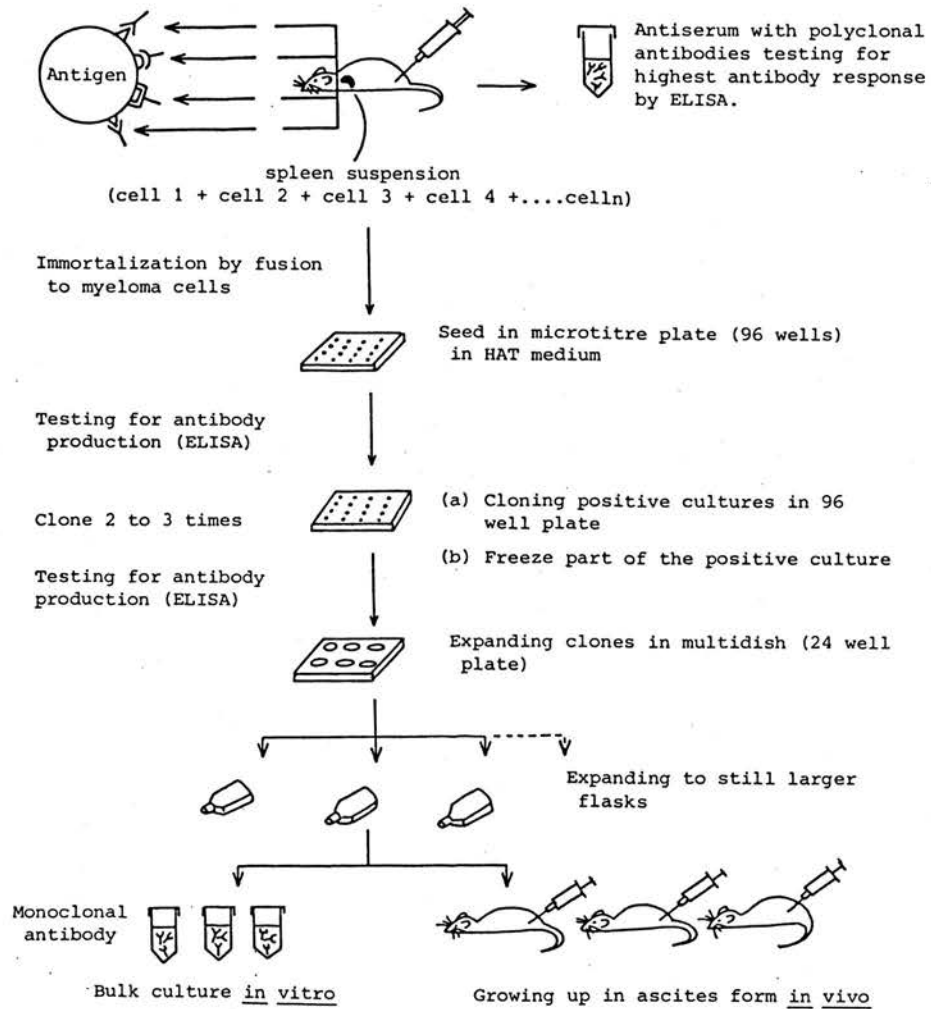
6.7.4 Subsequent karyotypic and cell surface phenotype analysis revealed that ES-12 cell line was a spontaneous transformant of an EBV-positive cell.

6.7.5 The antibody produced by ES-12 was of the IgG<sub>3</sub>, kappa isotype and protected mice from challenge with tetanus toxin.

6.7.6 Both the mouse and human antibody preparations appeared to react with the  $\alpha$  and  $\beta$  chain of tetanus toxin.

FIG. C.6-1a

HYBRIDOMA THEORY (RODENT SYSTEM)

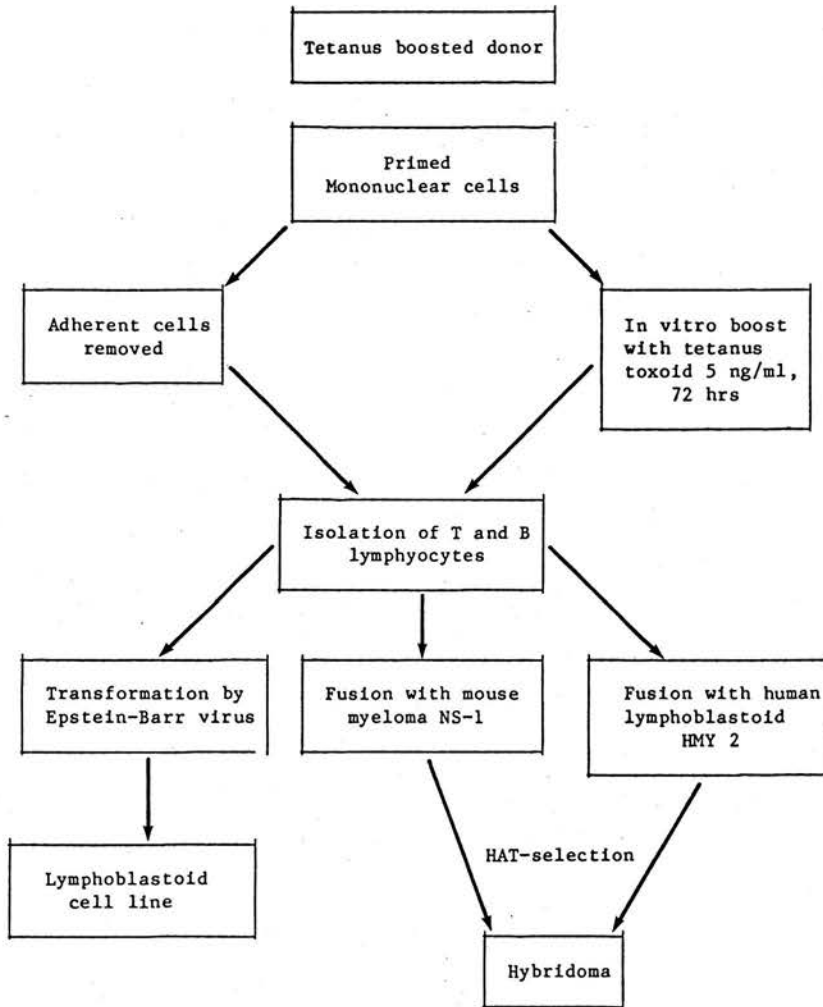


Principal steps involved in producing monoclonals of rodent origin.



FIG. C.6-1b

PRODUCTION OF MONOCLONAL ANTIBODY OF HUMAN ORIGIN



Principal steps involved in producing monoclonals of human origin.

TABLE C.6-1

THE PRODUCTION OF MOUSE MONOCLONAL ANTI-TETANUS ANTIBODIES

Fusion No.	Immunogen (tetanus-toxoid)	Days mice Injected	Day of Fusion	No. of Cultures	% with Hybrids	% Positive Hybrids
1	Wellcome	0,30	33	240	13	12
2	Connaught	0,30	33	300	5	0
3	Wellcome	0,28,56, 67	70	300	86	0.4
4	Connaught	0,28,56, 67	70	300	75	0
5*	Wellcome	0,28,56, 67	70	170	80	0.5
6*	Connaught	0,28,56, 67	70	160	54	0
7	Wellcome	0,14	17	480	23	0
8	Connaught	0,14	17	480	0	0
9	Wellcome	0,28,42	45	300	13	0
10	Connaught	0,28,42	45	300	21	15
11	Wellcome	0,30, (1 yr)	1 yr & 3 days	240	22	13

\* T - cell depleted spleen used in fusion.

TABLE C.6-2

ANTI-TETANUS Ab LEVELS OF MOUSE ASCITES FLUID DETECTED BY ELISA  
FROM FUSION 1 (Fl.6)-(UNCLONED CELL LINE)

Mouse No.	Days mouse tapped (post-injection of hybrid)	Ab-titre
1	7 10 11 12	1:160 1:320 1:640 1:640
2	7 8 9	1:40 1:320 1:640
3	7 8	1:40 1:80
4	7 9	1:80 1:320
5	7 9 14 16	1:40 1:20 1:160 1:320
Positive Control	Polyclonal mouse anti-tetanus serum	1:25600
Negative Control	Normal mouse serum	0

N.B.: Ab-titre of the original hybrid before inoculation was 1:256

TABLE C.6-3

ANTI-TETANUS Ab LEVELS OF MOUSE ASCITES FLUID DETECTED BY ELISA  
FROM FUSION 1 (F1.1)-(UNCLONED CELL LINE)

Mouse No.	Days mouse tapped (post-injection of hybrid)	Ab-titre
1	7 9 10	1:1280 1:2560 1:2560
2	7 8 9 11 12 13	1:40 1:80 1:160 1:160 1:320 1:320
3	7 8 9 10 11 12	0 1:40 1:80 1:160 1:320 1:320
Positive Control	Polyclonal mouse anti-tetanus serum	1:25600
Negative Control	Normal mouse serum	0

N.B.: Ab-titre of the original hybrid before inoculation was 1:256.  
 Mouse No.1 showed a 10 fold increase in the Ab-titre.

TABLE C.6-4

ANTI-TETANUS LEVELS OF MOUSE ASCITES FLUID DETECTED BY ELISA  
 FROM FUSION 1 (F1.1.2)-(CLONED CELL LINE)

Mouse No.	Days mouse tapped (post-injection of hybrid)	Ab-titre
1	7	1:320
	9	1:320
	10	1:640
	12	1:2560
	13	1:2560
	16	1:5120
	18	1:10,240
	20	1:10,240
2	7	1:320
	9	1:640
	11	1:640
	12	1:2560
	13	1:5120
	15	1:10,240
	18	1:10,240
	22	1:10,240

The above Ascites fluid containing high titre of monoclonal antibodies were given to Dr.S. Van Heyningen (Biochemistry Department) for use in their studies of molecular structures of tetanus toxin.

The antibody titre of the hybrids supernatants prior to injection was 1:256. The antibody activity increased on subsequent tapping, and the monoclonal antibody titres were 40 x higher than observed in culture supernatant.

TABLE C.6-5

ANTI-TETANUS Ab LEVELS OF MOUSE ASCITES FLUID DETECTED BY ELISA

FROM FUSION 1 (F1.1.3) — CLONED (2x) CELL LINE

Cell line	Days mouse tapped (post-injection of hybrid)	Ab-titre
1/7/3	7	1:1280
	8	1:2560
	9	1:5120
1/7/8	7	1:320
	9	1:320
	10	1:1280
1/7/12	8	1:20
	9	1:20
	10	1:40
	12	1:80
1/7/17	7	0
	8	0
1/9/6	8	1:160
	10	1:160
	13	1:640

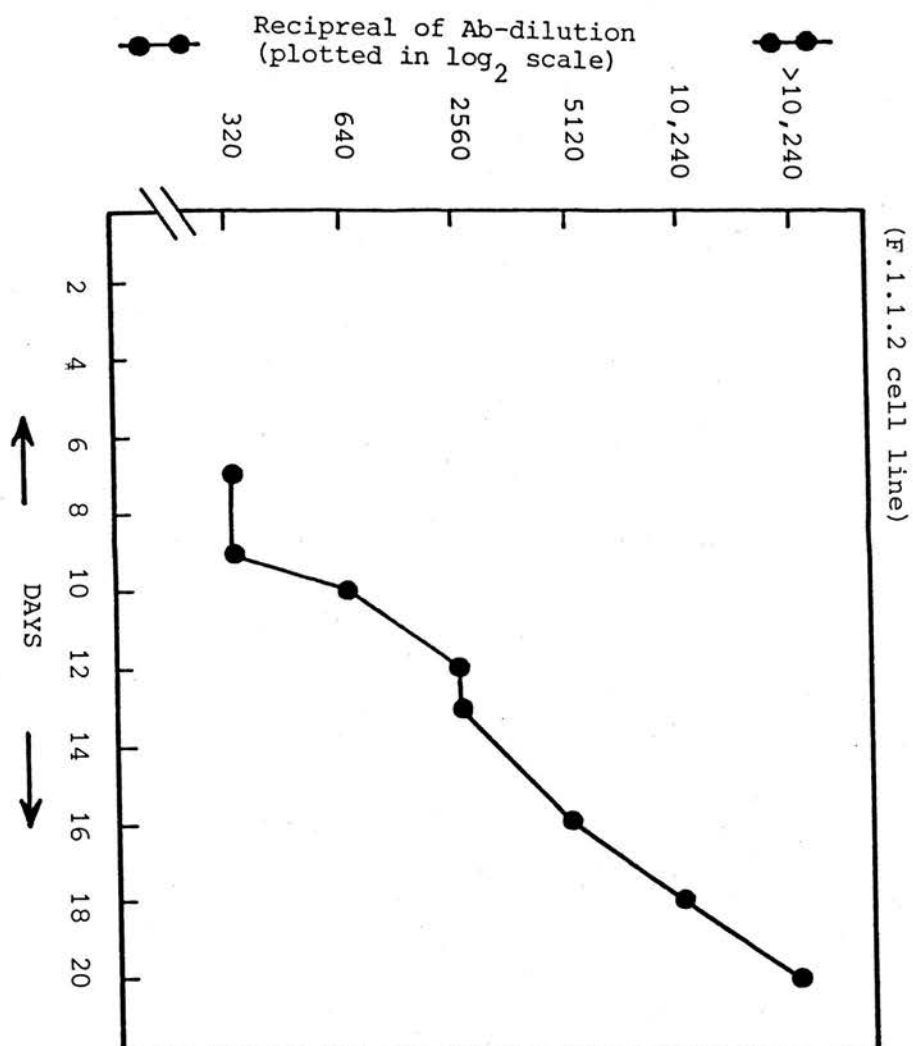
N.B. The Ab-titre of the original hybrids prior to inoculation was 1:160. The antibody was concentrated in ascitic form in various degree, ranging from 0-32 fold of the original.

FIG. C.6-2

When sequential tapping of ascitic fluid was performed, more concentrated antibody was obtained, and in most cases the Ab-titre increased linearly with subsequent tapping.

FIG. C.6-2

ANTI-TETANUS ANTIBODY LEVELS IN MICE ASCITIC FLUID TAPPED AT  
DIFFERENT INTERVALS AFTER INOCULATION WITH HYBRIDS





FIGS. C.6-3 and C.6-5

Profile of Protein-A Sepharose fractionated mouse ascitic fluid for isolation of the monoclonal antibody to tetanus. Almost all of the tetanus antibody activity (by ELISA o - - o) was observed in the IgG<sub>1</sub> peak.

FIGS. C.6-4 and C.6-6

Further analysis of the separated fractions (of Figs. 3 & 5) with isotype specific antisera using SEI assay confirmed that the monoclonal antibody to tetanus was of the IgG<sub>1</sub> class.

FIG. C.6-3

PROTEIN - A SEPHAROSE AFFINITY CHROMATOGRAPHY OF MONOCLONAL-Ab  
TO TETANUS TOXOID (ASCITIC FLUID) FROM FUSION 1

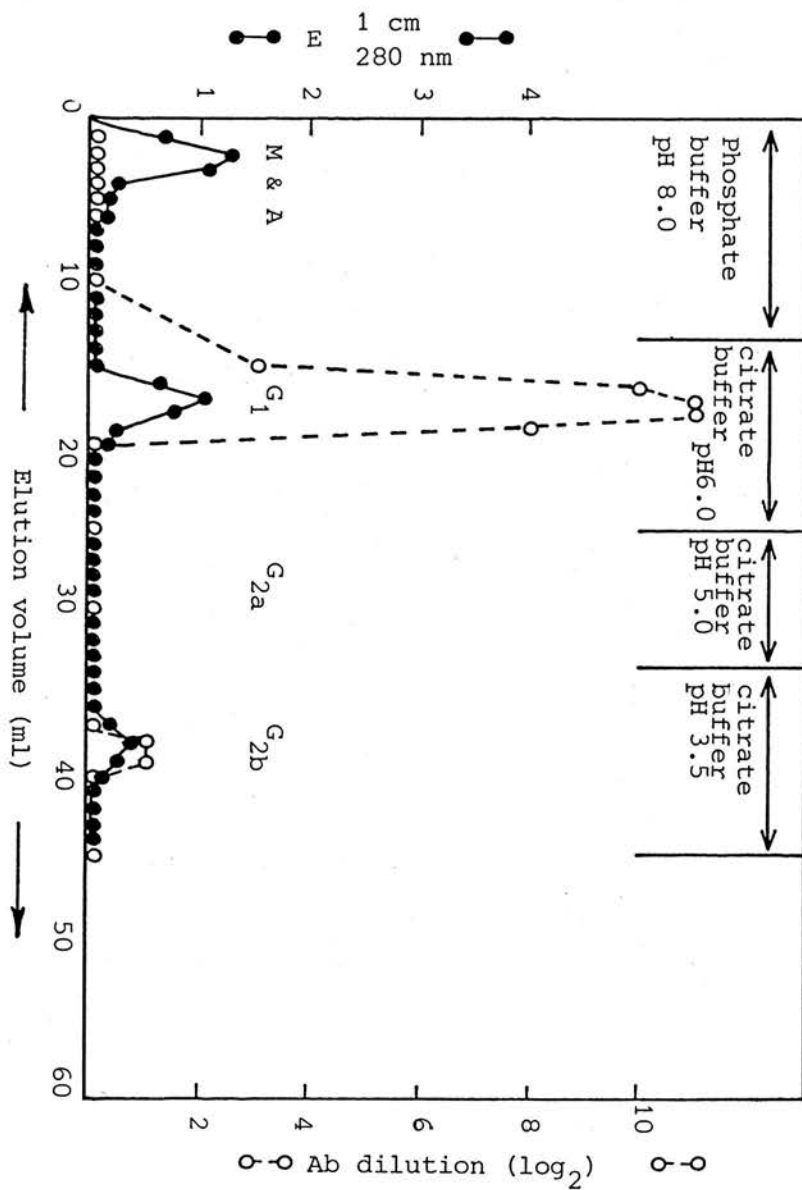


FIG. C.6-4

THE ISOTYPES OF MONOCLONAL ANTIBODY TO TETANUS TOXOID IN PROTEIN-A  
SEPHAROSE FRACTIONS OF ASCITIC FLUID  
(Fusion 1)

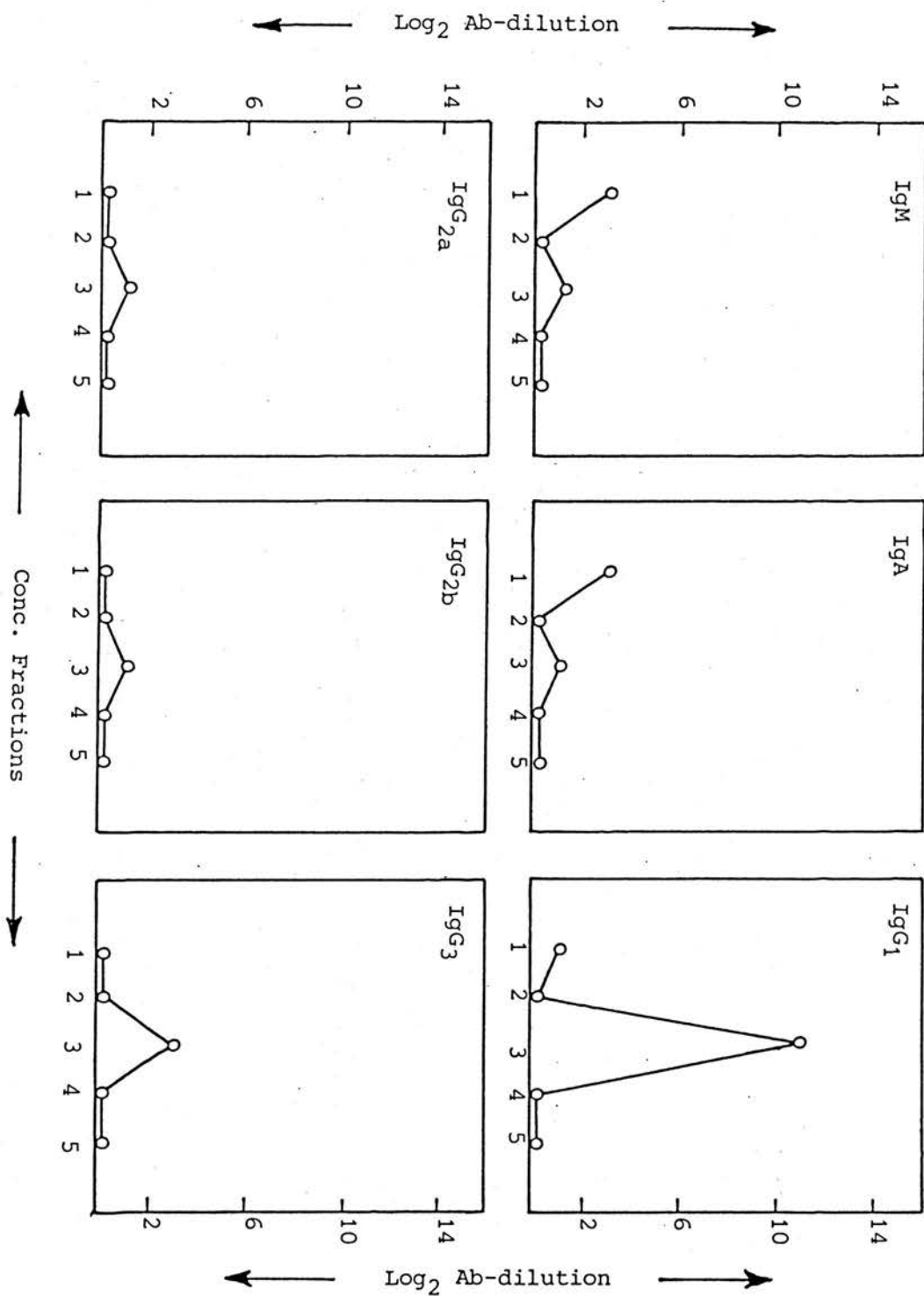


FIG. C.6-5

PROTEIN - A SEPHAROSE AFFINITY CHROMATOGRAPHY OF MONOCLONAL -  
AB TO TETANUS TOXOID (ASCITIC FLUID)  
(FUSION 6)

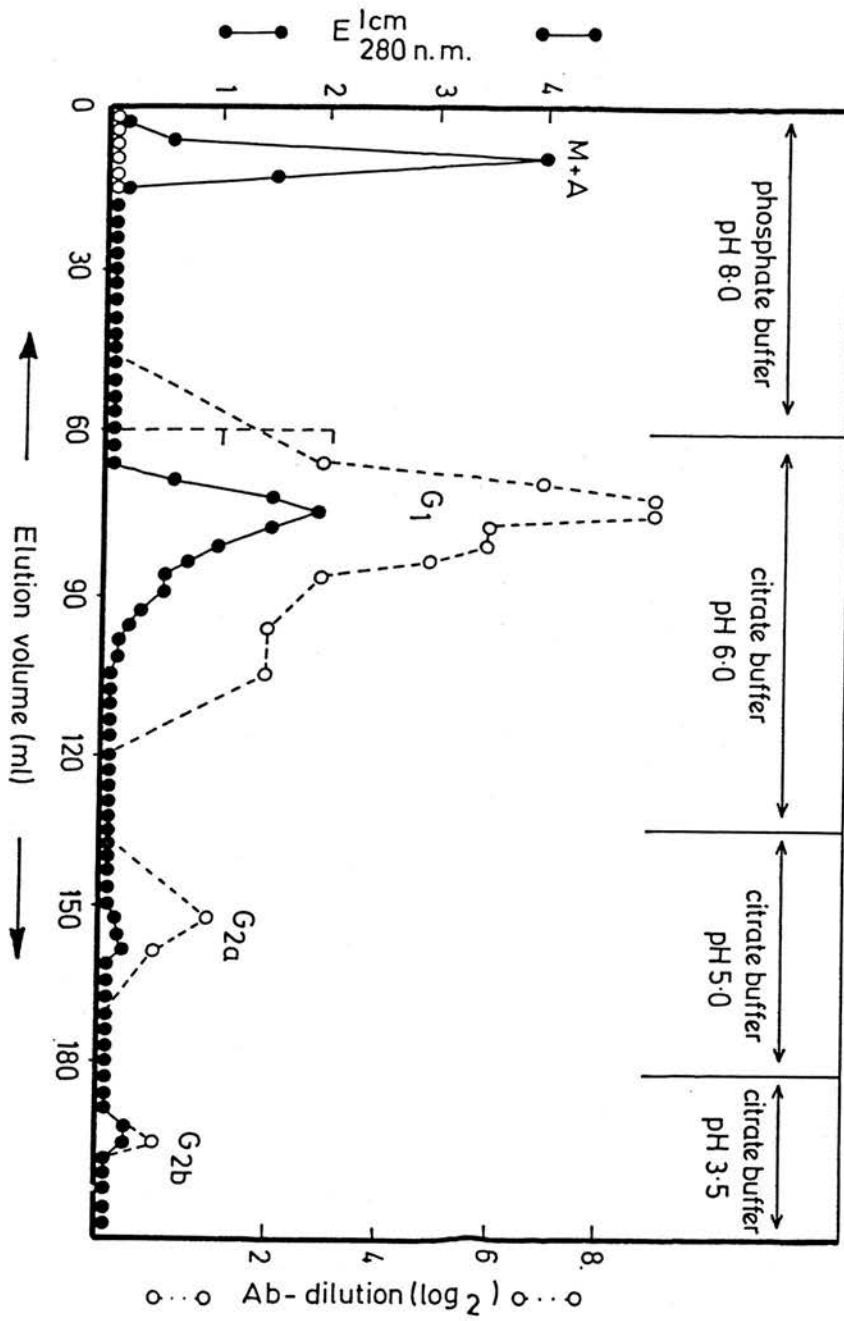


FIG. C.6-6

THE ISOTYPE OF ANTITETANUS ANTIBODIES IN PROTEIN - A FRACTIONS  
ASCITIC FLUID (CONNAUGHT - T.1)

(FUSION 6)

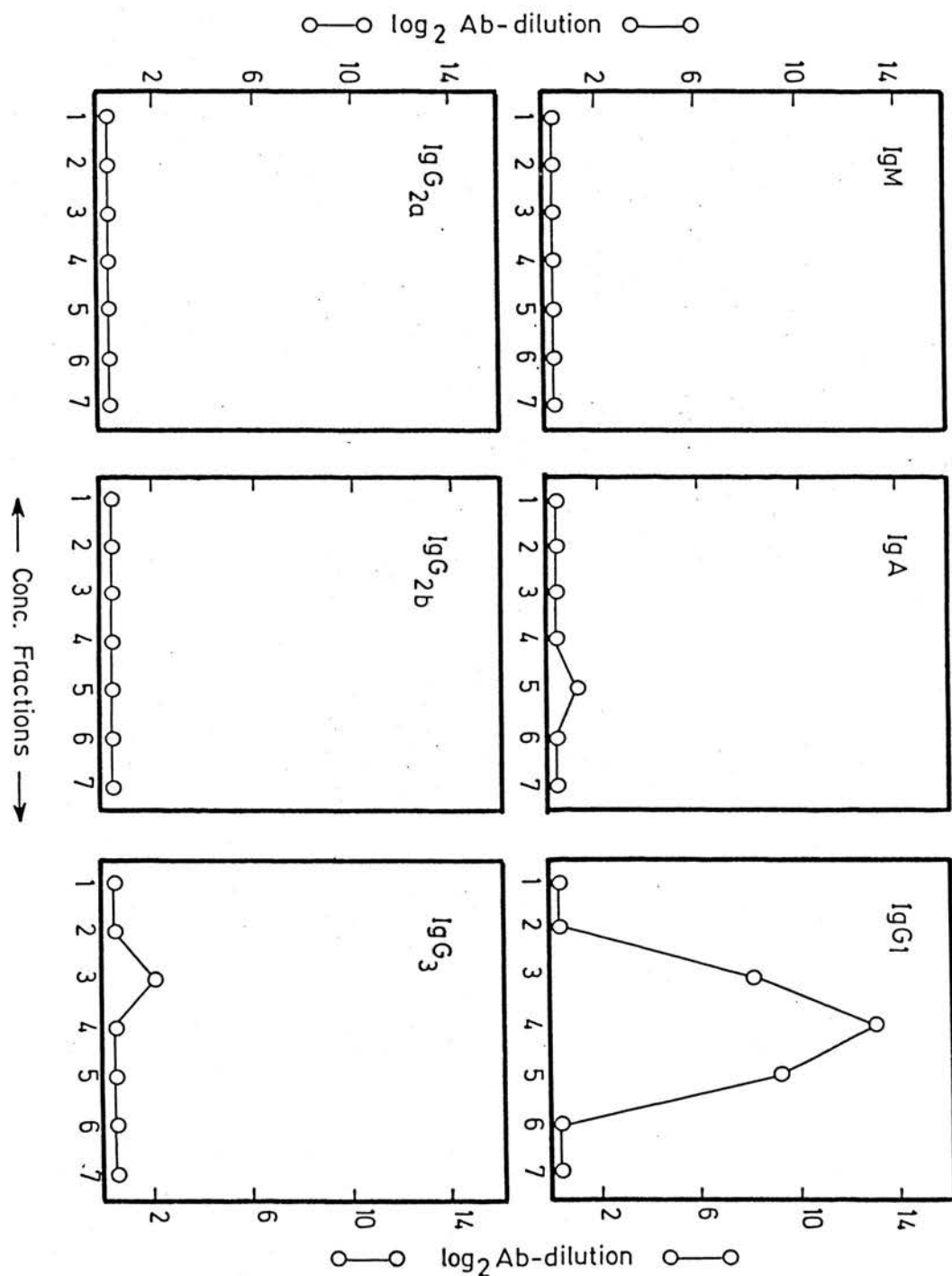


TABLE C.6-6

THE PRODUCTION OF HUMAN MONOCLONAL ANTIBODY TO TETANUS BY  
EBV-TRANSFORMATION OF LYMPHOCYTES

Fusion No.	Antigen (TT) boost	No. of cultures	No. with growth	No. positive in anti-tetanus activity
HV1	in vivo (a)	960 wells 20 pots	0	0
HV2	in vivo	1920 wells 5 pots	1 well 5 pots	2
HV2	in vivo	480 wells (cloned)	330 wells	8
HV6	- (b)	120 wells 5 pots	4 wells 5 pots	7
HV6	-	480 wells (cloned)	140 wells	4
HV7	in vivo + in vitro	120 wells 4 pots	4 pots	4

N.B. (a) Lymphocyte donors were boosted 7 days earlier

(b) Three months since the last boost

All cultures ceased to grow further

TABLE C.6-7

THE PRODUCTION OF HUMAN MONOCLONAL ANTIBODY TO TETANUS TOXOID USING MOUSE  
MYELOMA (NS-1) AND HUMAN LYMPHOBLASTOID (HMY 2) AS FUSION PARTNERS

Fusion No.	Fusion partner	Antigen TT boost	No. of cultures	No. with growth	No. positive in anti-tetanus activity
HF1	NS-1	—*	120 wells	2	0
HF4	NS-1	in vitro	120 wells 18 pots	0	0
HF5	NS-1	in vivo + in vitro	180 wells 12 pots	6	1
HF2 (=ES.12)	H MY 2.3	—*	480 wells	55	3
HF6	H MY 2.3	in vitro	238 wells 21 pots	43	1
HF3	H MY 2.9	—*	240	16	1
HF7	H MY 2.9	in vitro	240	3	0

N.B. \*Lymphocyte donors were boosted three months earlier.

All cultures except HF2 ceased to grow. The cell line derived from this fusion (designated ES-12) has continued to grow and secrete specific antibody.

TABLE C.6-8

LEVELS OF ANTITETANUS ANTIBODY IN DONOR'S SERUM TAKEN AT  
DIFFERENT INTERVALS

Donor	Date of Sample Collection	Haemagglutination		ELISA	
		Ab Titre	I.U.	Ab Titre	I.U.
1	Pre Boost	1:4	0.06	1:4	0.06
	Day 5	1:4	0.06	1:8	0.13
	Day 7	1:8	0.13	1:256	4.0
	Day 14	1:512	8.0	1:1024	16.0
	Day 26	1:256	4.0	1:1024	16.0
	6th month		2.0		16.0
2	Pre Boost	1:8	0.13	1:16	0.25
	Day 5	1:8	0.13	1:16	0.25
	Day 7	1:16	0.25	1:64	1.0
	Day 14	1:128	2.0	1:1024	16.0
	Day 26	1:128	2.0	1:1024	16.0
	6th month		2.0		16.0
3	Pre Boost	1:64	1.0	1:64	1.0
	Day 5	1:128	2.0	1:256	4.0
	Day 7	1:128	2.0	1:2048	32.0
	Day 14	1:1024	16.0	1:2048	32.0
	Day 26	1:1024	16.0	1:2048	32.0
	6th month		8.0		32.0
Tetanus IgG (PFC)	Batch No.18	1:16000	250	1:32000	250
Tetanus IgG (PFC)	Batch No.17	1:8000		1:16000	
Negative Control	Normal human serum	0	0	0	0

N.B. Ab-response varied among individuals but the peak of response was day 7 or 14 after booster immunization



#### D. CONCLUDING DISCUSSION

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The main purpose of the work presented in this thesis was to devise alternative techniques for isolation of antibodies to tetanus toxin, and possibly to other biologically active agents.

In the initial phase, to ensure the proper selection of donors for the production of tetanus-antibodies, a sensitive and simple assay which would permit the measurement of circulating anti-tetanus antibodies in man and mouse, and in the specific antibody preparations derived from the plasma or hybridoma cell lines was required. The ELISA assay was chosen to be developed in preference to equally sensitive radioimmunoassay (RIA) for a number of reasons. These include: its greater rapidity, lower cost, longer shelf life of reagents as well as safety reasons. The most valuable findings were that microtitre plates coated with tetanus toxoid were found to have a shelf life of more than one year with no reduced sensitivity. The assay was found to be extremely sensitive, capable of measuring an anti-tetanus antibody level as low as  $5 \times 10^{-4}$  I.U./ml. The lowest tetanus antibody levels measured by other techniques are: 6 I.U./ml by radial immunodiffusion (Eldridge et al., 1975 and Cook et al. 1976), 5 I.U./ml by counterimmunoelectrophoresis (Entwistle et al. 1973), 0.004 - 0.016 I.U./ml by RIA (Dow et al. 1983, Habermann, 1977 and Bernath, 1974).

All in vitro methods for measurement of tetanus antibody are generally less specific than in vivo neutralization tests, and the main problem has been to show that there is a good correlation between the two tests. In a comparative study testing our human monoclonal antibody to tetanus, both the mouse protection assay and the ELISA

showed similar antibody titre (2.5 - 3.0 I.U./ml). The neutralizations method is however expensive, time-consuming and requires a large number of suitable animals. A further limitation is that the method also requires a relatively large volume of sample, especially when the antibody concentrations are low. The sensitivity of the toxin neutralization test in mice was recently reported to be only 0.01 I.U./ml, the minimum level thought sufficient for protection.

In the initial studies the ELISA was compared with haemagglutination assay (HA) in which toxoid-coated human red blood cells were used in preference to the sheep, turkey, goose and duck red cells used by others (Konstantinova, 1981 and Pitzurra, 1981). Although this HA test was faster than ELISA and was of adequate sensitivity, it was less reproducible and amenable to automation and standardization than the ELISA method. Furthermore, the HA test will measure the tetanus-IgG only in the absence of IgM antibodies (Crawford et al. 1980). This ELISA technique is now being used routinely by the Blood Transfusion Service (S.E. Scotland and Edinburgh) for measuring the anti-tetanus antibody levels in the plasma of donors, and by the Department of Surgery (University of Edinburgh) for screening the monoclonal antibodies to tetanus.

The experiment using the ammonium sulphate precipitation technique to measure the binding capacity and binding affinity of anti-tetanus antibody proved to be irreproducible. A number of experiments were undertaken to investigate factors that could have been responsible for this. While these investigations failed to resolve the problem, it is possible that an alternative labelling technique (see B.3) or precipitation reagent (Tanavala et al. 1978), and the use of highly purified tetanus toxoid (see Chapter 3) might overcome some of these problems.

The studies on tetanus toxoid preparations revealed that Wellcome tetanus vaccine is extremely heterogeneous. The protein peak containing most of the toxoid activities is 4.5 S. This observation is in agreement with Pillemer et al (1948) and Largier (1956), but in contrast with Raynaud et al. (1960) and Murphy et al. (1967). The highly pure tetanus toxoid (Conn-TT) is homogeneous and consists of only one main protein peak (4.5 S) containing all of the toxoid antigenicity.

It seems that formaldehyde treatment of tetanus toxin in order to prepare toxoid leads to significant polymerization (Bizzini et al. 1974 and Murphy, 1967). This could explain the different values reported for the size of tetanus toxoid molecules. The allergy reactions following active immunization for tetanus are believed to be due to impurities present in the preparation, and to the polymerization resulting from formaldehyde treatment (Wellhöner, 1981). It appeared that the Conn-TT which proved to be the purest of toxoid preparations may have been purified prior to toxoiding. This tetanus toxoid also radiolabelled with iodine more efficiently. Whether or not this pure preparation is more effective at eliciting anti-tetanus antibodies in human as it does in murine, or produces fewer or weaker side reactions, remains to be established.

The method initially adopted to extract antibodies with specificity to tetanus toxin from human plasma, and from standard commercial immunoglobulins (Cohn Fraction II), was affinity chromatography. The technique utilizes the unique specificity of antigen-antibody interaction. This approach is especially useful when isolating specific antibodies present rarely or at low concentration.

A number of studies had to be undertaken to establish the optimum conditions for the preparation of immunoadsorbent columns. Various factors with respect to antigen (tetanus toxoid) concentration, temperature, incubation time, coupling buffer, and age of the sepharose were evaluated, and were found to influence the binding (see Chapter 5). The column so prepared had an excellent chemical stability and proved economical. The same column was used repeatedly to isolate anti-tetanus antibody without loss of capacity or specific properties. It could be concluded that the antigenic sites of the column were not either blocked, inactivated or eluted as a result of successive cycles.

All the elutions were carried out at room temperature, and antibody elution was performed at acid pH without loss of biological activity. The purification factor achieved was as high as 108-fold, which is at least four times higher than obtained by the Cohn-fractionation technique (Krijnen et al. 1970 and Levine et al. 1967). The specific neutralizing activity of the final product was 80-180 I.U. per mg or protein depending on the specific activity of the starting material. This is at least twice as high as found by Tardy et al. (1978), who used porous silica beads for the purification of tetanus-antibody.

One of the main differences between this technique and the classical column methods is its extreme rapidity, a factor which helps minimize denaturation. Using this technique, almost all of the specific antibody can quickly be isolated in not more than 1% of the starting immunoglobulin. This permits the production of a purified antibody preparation of high specific activity, so that it is possible to give a large dose of antibody in a small volume containing

relatively little protein. Such preparations are likely to be safe to administer by intramuscular, intravenous and intrathecal routes.

This immunoaffinity technique is widely applicable. There are good reasons to believe that this approach could be used (as it was intended) successfully for isolation of other antibodies of potential clinical value, including anti-Hepatitis B surface antigen, anti-Varicella-Zoster, anti-Cytomegalovirus and anti-Pneumococcal polysaccharide.

The immune response studies undertaken in mice resulted in some interesting findings. The analysis of the Ig-isotypes were done by SEI assay developed for this study. The responses of the mice to both toxoid preparations are largely in the IgG isotype. This finding was later confirmed by Ershler et al. (1982) who found that mice produced a poor primary response to tetanus toxoid and, on boosting the antibody was mainly IgG. However, in the present studies, antibody responses were detected in all IgG subclasses except IgG<sub>2b</sub> immediately after primary challenge with pure tetanus toxoid (Conn-TT). This toxoid preparation not only elicited rapid, but also a more pronounced response than the crude Wellcome vaccine.

An interesting finding was the presence of "naturally acquired" antibodies in conventionally housed CBA/Ca and BALB/c mice. Similar findings have recently been reported in non-vaccinated humans and animals in the Galapagos Islands (Veronesi et al. 1983), and in India (Wellhöner, 1981). No reports have been available on the Ig-isotypes of these antibodies. According to the results presented here (Chapter 4) the pre-existing antibodies to tetanus are of the IgA, IgM and to a lesser extent of the IgG<sub>3</sub> isotypes. The fact that the natural antibodies were found against both crude and pure toxoids indicates

that the antibodies were against the tetanus toxoid and not a contaminating protein. Both strains of mice exhibited similar responses.

It is possible that spores of Cl. tetani are swallowed by man and mice, and have stimulated a response. It has recently been suggested that the oral route might provide an effective vaccination route for humans against tetanus (Veronesi et al. 1983). This route might preferentially stimulate IgA and IgM antibodies. Alternatively, determinants might be shared between tetanus toxoid and other macromolecules or intestinal organisms such as other Clostridia. It would be of great interest to verify whether a single dose of tetanus toxoid would boost these low titres of "natural antibodies" to a protective level in humans. This is significant where the present three doses scheme given at spaced intervals does not receive the cooperation of a large proportion of the population. Recently in Denmark in those who had received only primary vaccination an exponential fall off in immunity was seen, and 25-30 years after primary vaccination, 28% had serum antitoxin concentrations below ( $<0.01$ ) the level of protection (Simonsen et al. 1984).

The studies undertaken on the immune response of mouse to tetanus toxoids were also important preliminaries to our eventual aim of producing a suitable anti-tetanus antibody of specific Ig-isotype for clinical use, either by immunization of blood donors or by the monoclonal procedure.

The in vitro production of McAb which had become popular during my course of study, seemed to be another attractive approach to our aim of the production of "specific" tetanus antibody. McAb which have been called "the smart bombs of biology" (cited by Brown, 1982), normally

bind to a single epitope on a single antigen. The major advantage of this technology is that it enables the production of a continuous supply of pure antibody of precisely defined specificity and isotype.

Production of McAb to tetanus toxoid in both mouse and human was successful. The mouse hybrids were grown up in both tissue culture and ascites form. By performing sequential taps of ascites-bearing mice, anti-tetanus antibody preparations of high titre were obtained. The mouse monoclonals were of the IgG<sub>1</sub> isotype by both Protein-A Sepharose fractionation and the SEI techniques. IgG<sub>1</sub> was just recently found to be the predominant subclass of in vivo and in vitro produced mouse anti-tetanus antibodies (Lutz et al. 1983). The level of antibody activity of one of the mouse cell lines was low, and eventually dropped to zero. It is apparent that the hybrid has either lost the relevant gene or has been overgrown by non-antitetanus secreting hybrids. Decrease or loss of secreted antibody has been reported by others (Zurawski et al. 1978).

A relatively stable human cell line (designated ES-12) secreting anti-tetanus antibody was obtained following the fusion of peripheral blood lymphocytes from an immunized volunteer with a human lymphoblastoid cell line (HMY 2.3). Subsequent karyotype analysis revealed that this was a spontaneous transformant rather than a true hybrid. The antibody produced by ES-12 protected mice from challenge with tetanus toxin.

The frequency of the human-mouse hybrids were very low, and they tended to be less stable than their human counterparts. Although initially there was a vigorous growth of EBV-transformed cell lines which produced high levels of specific antibodies, they ceased to grow after 9 months in culture and proved difficult to clone, possibly due



to loss of chromosomes (Zurawski et al. 1978).

These monoclonal antibodies, produced by both mouse and human (ES-12) cell line, have been shown by immunoblotting technique to bind to particular peptides in the digests derived from both chains of tetanus toxin (Van Heyningen, personal communication). This observation is of importance with respect to the evolution of the tetanus toxin molecule suggesting it arose by gene duplication. Studies of tetanus toxin compositions would support such a view, as there is a sequence homology between the heavy and light chain (Cornish-Bowden, 1983 and Taylor et al. 1983).

Of additional interest are recent reports suggesting that at least two antigenic determinants of tetanus toxin molecule should be blocked by antibodies for efficient neutralization, and this is believed to require at least two monoclonal antibodies directed against different epitopes (Mizuguchi et al. 1982). Moreover, a recent report shows that monoclonal antibodies to tetanus have appeared to be less suitable than polyclonal sera for protection in animals (Ahnert-Hilger et al. 1983). Such reservations are not fully supported by those in our own laboratories.

The tetanus monoclonal antibodies are attractive tools which would allow one to extend and specify the structure-function relationship of different molecules of toxin. From the therapeutic viewpoint it seems that the use of antibodies directed against the whole toxin molecule is to be preferred for the time being to that of antibodies directed to particular parts of toxin molecule (Bizzini, 1981).

While the advantages of monoclonal antibodies are considerable, some caution is merited, particularly relevant to clinical usage. These include the high cost of production, the immunogenicity

of monoclonal antibodies of murine origin, human antibodies could stimulate anti-idiotypic antibody responses and finally there is the possible hazard of transferring oncogenic material. Nevertheless, with the technology in this field and in the field of gene cloning evolving so rapidly, the future for human monoclonals holds great promise, especially with the recent concern over the safety of blood production in regard to the newly found blood transmissible disease AIDS.

It would be of great interest to compare the biological effectiveness of the human tetanus antibodies produced by hybrids with the immunoaffinity purified preparations, which have wider biological flexibility both in molecular classes and in specificity.

In view of the comments expressed in many articles on tetanus-serotherapy (Lancet editorials, 1974, 1980 and October 27th, 1984), further study in this area is clearly wanted. However, in spite of the recent advances in antibody production and purification it remains to be shown that these novel products will be of any greater clinical value than those currently in existence. Nevertheless, it is my firm belief that optimism in products of this nature is fully justified.

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